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Summary Sentence
Melatonin activates sperm adhesiveness and affects motion patterns of boar spermatozoa subjected to in vitro capacitation. These effects were not apparently linked to a direct antioxidant action, although they were related to the maintenance of proper levels of intact disulphide bonds.

Summary

This work sought to address the effects of melatonin during in vitro capacitation (IVC) and progesterone-induced acrosome exocytosis (IVAE) in boar spermatozoa. With this purpose, two different experiments were set. In the first one, IVC and IVAE were induced in the absence or presence of melatonin, which was added either at the start of IVC or upon triggering the IVAE with progesterone. Different parameters were evaluated, including intracellular levels of peroxides and superoxides, free cysteine radicals and distribution of specific lectins. While melatonin neither affected most capacitation-associated parameters nor IVAE, it dramatically decreased sperm motility, with a maximal effect at 5 µm. This effect was accompanied by a significant increase in the percentage of agglutinated spermatozoa, which was independent from noticeable changes in the distribution of lectins. Levels of free cysteine radicals were significantly lower in melatonin treatments than in the control after 4 h of incubation in capacitating medium. The second experiment evaluated the effects of melatonin on in vitro fertilising ability of boar spermatozoa. Spermatozoa

previously subjected to IVC in the presence of 1 μm melatonin and used for in vitro fertilisation exhibited less ability to bind the zona pellucida (ZP) and higher percentages of monospermy. In conclusion, melatonin affects sperm motility and the stability of nucleoprotein structure and also modulates the ability of in vitro capacitated boar spermatozoa to bind the oocyte ZP. However, such effects do not seem to be related to either its antioxidant properties or changes in the sperm glycocalix.

Introduction

Mounting evidence supports the existence of a functional machinery related to melatonin metabolism in the mammalian reproductive tract (Reiter et al., 2009). While melatonin receptors MT1 and MT2 are present in the spermatozoa from humans, hamsters, pigs, dogs, cattle, deer and sheep (González-Arto et al., 2016), they are absent from other species, such as horses (Da Silva et al., 2011). These two receptors are detected in both seasonal and non-seasonal species, and their presence is concomitant with that of melatonin in the seminal plasma (concentration range: 0.5–5 μm; Luboshitzky et al., 2002; Casao et al., 2010; Pérez- Patiño et al., 2016). All these data suggest the existence of an active melatonin pathway system in mammalian spermatozoa.

The main role of melatonin has usually been linked to the regulation of circadian rhythms, including those related to the reproductive function (Reiter et al., 2009). However, while the presence of melatonin in seminal plasma and MT receptors in spermatozoa has been clearly associated with circadian modulation in seasonal breeders, such as the sheep (Casao et al., 2010), its relationship with the circadian rhythm in non-seasonal breeders, such as the pig, seems to be dismissed (González-Arto et al., 2016).

Focusing on the effects upon sperm function, melatonin seems to have a vital regulatory role for sperm capacitation in the sheep and water buffalo (Casao et al., 2009; Ashrafi et al., 2013). Furthermore, melatonin has been shown to improve motility and other sperm functional parameters in human, ram, equine and boar spermatozoa (reviewed in Cebrián-Pérez et al., 2014). The mechanism through which melatonin exerts these effects has been suggested to be linked with a reduction in oxidative stress by scavenging intracellular free radicals (Reiter et al., 2000). In agreement with this hypothesis, in vitro treatment of spermatozoa with melatonin decreases intracellular levels of reactive oxygen (ROS) and nitrogen species (RNS; Rao & Gangadharan, 2008; Du Plessis et al., 2010; Jang et al., 2010; Najafi et al., 2018), membrane lipid peroxidation (Gadella et al., 2008; Du Plessis et al., 2010; Da Silva et al., 2011), apoptosis markers (Casao et al., 2010; Da Silva et al., 2011; Espino et al., 2011; Najafi et al., 2018) and DNA fragmentation (Sarabia et al., 2009). In spite of all these data, little is known about whether melatonin could exert any effect on sperm function through a mechanism independent from its antioxidant properties. This is especially relevant for some species such as the pig, in which ROS levels produced by their spermatozoa in response to cryopreservation are marginal when compared to other species, such as the horse and cattle (Bilodeau et al., 2000; Guthrie & Welch, 2006; Yeste et al., 2013, 2015a,b). Thus, ROS production or accumulation seems to play a minor role to explain specific events, such as boar sperm cryodamage (Yeste et al., 2013, 2015b). Likewise, changes in ROS levels are also low during the achievement of in vitro boar sperm capacitation (IVC), which again suggests they play a marginal role (Awda et al., 2009). Taking all these data into account, we can hypothesise that melatonin could affect boar sperm function through mechanisms other than its ability to modulate intracellular ROS levels.

This study sought to determine the influence of melatonin on the achievement of IVC and subsequent progesterone-induced in vitro acrosome exocytosis (IVAE) of boar spermatozoa, as well as on their ability to adhere and further penetrate in vitro maturated oocytes. With this purpose, two experiments were devised. In the first one, boar spermatozoa were subjected to IVC/IVAE in the presence of

increasing concentrations of melatonin, added either before or after 4 h of incubation under capacitating conditions. Several parameters related to the achievement of IVC and IVAE were evaluated. In the second experiment, in vitro fertilisation (IVF) was conducted with spermatozoa previously capacitated with 1 μm melatonin. The sperm ability to bind the oocyte ZP and penetrate in vitro matured oocytes was assessed.

Materials and Methods

Seminal samples

A total of 57 ejaculates collected from 32 healthy Pietrain boars aged between two and three years were used. These animals were housed in climate-controlled commercial farms (Servicios Genéticos Porcinos, S.L., Roda de Ter, Spain), fed with a commercial adjusted diet and provided with water ad libitum. Boar housing fulfilled with the welfare standards established by European regulations on livestock species, specifically, on pig farms. Furthermore, and despite not being required as we did not manipulate any boar and only worked with seminal doses provided by the commercial farm, the experimental protocol was approved by the Ethics Committee of our institution (Bioethics Commission, Autonomous University of Barcelona; Bellaterra, Cerdanyola del Vallès, Spain). In all the cases, samples came from sperm-rich fractions that were obtained through manual collection with the conventional hand-gloved method. Upon collection, samples were immediately diluted with a commercial extender (Androstar Plus®; Minitub Ibérica SL, Tarragona, Spain) to a final sperm concentration of 2 × 107 spermatozoa/mL and cooled down to 16 °C. Diluted semen was packaged in 90-mL commercial AI doses and transported in an insulated container at 16 °C for approximately 45 min, which was the time required to arrive to our laboratory.

In vitro capacitation and progesterone-induced acrosome exocytosis

As aforementioned, two experiments were set. The first experiment was subdivided into two parts. The first one aimed at testing how melatonin affected the achievement of IVC (i.e. addition at 0 h). The second part sought to determine the impact of melatonin upon triggering IVAE in fully in vitro capacitated boar spermatozoa (i.e. addition at 4 h). With this purpose, different concentrations of melatonin were added after 4 h of incubation in CM, as this time period has previously been reported to induce IVC in boar spermatozoa (Ramió et al., 2008). In this case, the addition of melatonin was performed together with that of progesterone, which was used to induce the acrosome reaction (Jimenez et al., 2003; Wu et al., 2006). Regardless of when melatonin was added (i.e. either at 0 h or at 4 h), five different treatments were assayed: a positive control (C+), which consisted of spermatozoa incubated in CM containing bicarbonate and bovine serum albumin (BSA); three treatments with different concentrations of melatonin (0.5, 1, 5 μm) in CM; and a negative control (C−), which consisted of spermatozoa incubated in non-capacitating medium without bicarbonate or BSA (NCM). As aforementioned, the tested concentrations of melatonin were within the physiological range of the genital tract, as described in previous works (Luboshitzky et al., 2002; Casao et al., 2009, 2010; Ashrafi et al., 2013; Cebrián-Pérez et al., 2014; Pérez- Patiño et al., 2016).

For both parts, 50 mL of a given semen sample was split into five aliquots of 10 mL each. Aliquots were centrifuged at 600 g and 16 °C for 10 min and resuspended either with NCM (C−), CM (C+) or melatonin treatments (i.e. CM supplemented with melatonin at final concentrations of 0.5, 1 or 5 μm). In all cases, final sperm concentration was adjusted to 2 × 107 sperm/mL. The composition of NCM was 20 mm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) buffer (pH = 7.4), 112 mm NaCl, 3.1 mm KCl, 5 mm glucose, 21.7 mm sodium L-lactate, 1 mm sodium pyruvate, 0.3 mm NaHPO4, 0.4 mm MgSO4 and 4.5 mm CaCl2 (osmolarity: 287 mOsm/kg ± 6 mOsm/kg).

Capacitation medium (CM) consisted of NCM supplemented with 37.6 mm NaHCO3 and 5 mg/mL BSA (pH adjusted to 7.4; osmolarity: 304 mOsm/kg ± 5 mOsm/kg).

Aliquots were incubated at 38.5 °C and 5% CO2 in humidified air for 4 h either with or without melatonin, as described in Ramió et al. (2008). Samples were taken at 0 h and 4 h of incubation for analysis of sperm parameters. After 4 h of incubation, spermatozoa were subjected to progesterone-induced in vitro acrosome exocytosis (IVAE) through adding 10 mg/mL progesterone. After thoroughly mixing, sperm samples were incubated at 38.5 °C and 5% CO2 in humidified air for a further hour. Separate aliquots were taken at 1, 5 and 60 min after the addition of progesterone. In the case of the second part, melatonin was directly added to the corresponding tube at final concentrations of 0.5, 1 or 5 µm after 4 h of starting the experiment rather than at 0 h.

At each relevant time point (i.e. 0, 4 h, and after 1, 5 and 60 min of progesterone addition), the achievement of both IVC and IVAE was evaluated on the basis of the following parameters: sperm motility, agglutination, viability, acrosome exocytosis, membrane lipid disorder and tyrosine phosphorylation of P32 protein, as a specific capacitation marker of boar spermatozoa (Bravo et al., 2005). Furthermore, intracellular ROS levels, free cysteine residues in sperm head and tail extracts and lectin distribution over sperm membrane were also evaluated as parameters that could be related to the effects of melatonin on IVC/IVAE.

Unless stated otherwise, all fluorochromes and lectins were purchased from Molecular Probes® (Invitrogen, ThermoFisher; Eugene, OR, USA) and diluted with dimethyl sulfoxide (DMSO; Sigma-Aldrich; Saint Louis, MO, USA).

Evaluation of sperm motility and agglutination

Sperm motility and agglutination were evaluated by utilising a commercial, computer-assisted sperm analysis (CASA) system (Integrated Sperm Analysis System V1.0; Proiser, Valencia, Spain). This system is based on the analysis of 25 consecutive digitalised photographic images obtained from a single field at a magnification of 100× (Olympus BX41 microscope; Olympus 10 × 0.30 PLAN objective lens, negative phase contrast; Olympus Europa GmbH, Hamburg, Germany). These 25 consecutive photographs were taken at a time lapse of 1 sec, which implies that an image was captured every 40 ms. Five to six separate fields were taken for each replicate, and five replicates were evaluated per sample and treatment. Prior to evaluation with CASA, a 5 µL drop was placed onto a warmed Makler chamber (Sefi Medical Instruments, Haifa, Israel). In the case of samples evaluated at 0 h, they were previously warmed at 38 °C for 15 min in a water bath. Recorded sperm motility and kinematic parameters were those described in Ramió et al. (2008). Settings for the CASA system were as follows: area of particles: 10–80 µm2; curvilinear velocity (VCL): 1–500 µm/sec; mean velocity (VAP): 1–500 µm/sec; linearity coefficient (LIN): 10–98%; straightness coefficient (STR): 10–98%; mean amplitude of lateral head displacement (ALH): 0–100 µm; and beat cross-frequency (BCF): 0–100 Hz. A spermatozoon was considered to be motile when its VAP was higher than 10 µm/sec.

Furthermore, the number of spermatozoa included in agglutination complexes and the percentage of agglutinated spermatozoa that showed apparent tail movement were determined by evaluation of each consecutive photograph obtained from CASA analyses. Specifically, we determined the number of sperm heads agglutinated divided by the total number of sperm heads, and the number of beating tails observed in each agglutination complex divided by the total number of tails.

Flow cytometry analyses

Sperm viability, acrosome exocytosis, membrane lipid disorder and intracellular ROS levels were evaluated by flow cytometry. Information about flow cytometry analyses is given according to the recommendations of the International Society for Advancement of Cytometry (ISAC; Lee et al., 2008). Prior to evaluation, sperm concentration was adjusted to 1 × 106 spermatozoa/mL in a volume of 0.5 mL. Thereafter, spermatozoa were stained with the appropriate combinations of fluorochromes, following the protocols described below.

Samples were evaluated through a Cell Laboratory QuantaSC cytometre (Beckman Coulter, Fullerton, CA, USA) and were excited using single-line visible light from an argon laser (wavelength: 488 nm; power: 22 mW). Sheath flow rate was set at 4.17 µL/min, and electronic volume (EV; equivalent to forward scatter) and side scatter (SS) were recorded for each event. Calibration of the equipment was periodically performed using 10-µm Flow-Check fluorospheres (Beckman Coulter). Three optical filters with the following characteristics were used: FL1 (green fluorescence): Dichroic/Splitter, DRLP: 550 nm, band-pass filter: 525 nm, detection width 505–545 nm; FL2 (orange fluorescence): DRLP: 600 nm, BP filter: 575 nm, detection width: 560–590 nm; and FL3 (red fluorescence): long pass filter: 670 nm. Signals were logarithmically amplified, and photomultiplier (PMT) settings were adjusted to each particular staining method; compensation was used to minimise spillover of the fluorescence into a different channel. The analyser threshold was adjusted on the EV channel to exclude subcellular debris (particle diameter<7 µm) and cell aggregates (particle diameter>12 µm) and sperm-specific events were positively gated on the basis of EV/SS distributions. Three independent replicates were examined, and 10,000 events were evaluated per replicate. Information on the events was collected as list-mode data files (LMD), and data were analysed through Cell Lab QuantaSC MPL Analysis Software (version 1.0; Beckman Coulter).

In all assessments except SYBR14/PI, data obtained from flow cytometry experiments were corrected according to the procedure described in Petrunkina et al. (2010).

Evaluation of sperm viability

Sperm viability was assessed using the LIVE/DEAD® Sperm Viability Kit (SYBR14/ PI) following the protocol set in Garner & Johnson (1995). With this purpose, sperm samples were incubated with SYBR14 (final concentration = 100 nm) at 38 °C for 10 min and then with propidium iodide (PI; final concentration = 10 µm) at the same temperature for 5 min. Fluorescence emitted by SYBR14 was measured through FL1, whereas that emitted by PI was detected through FL3. Three sperm populations were identified as follows: (i) viable green-stained spermatozoa (SYBR14+/PI−); (ii) non-viable, red-stained spermatozoa (SYBR14−/PI+); and (iii) non-viable spermatozoa that were stained both in green and in red (SYBR14+/PI+). Non-sperm particles (debris) were found in the SYBR14−/PI− quadrant. Single-stained samples were used for setting PMT voltages of EV, FL1 and FL3, and for compensation of SYBR14 spillover into the FL3 channel (2.45%).

Evaluation of acrosome exocytosis

True acrosome exocytosis was determined through costaining of spermatozoa with Arachis hypogaea agglutinin (PNA) conjugated with fluorescein isothiocyanate (FITC-PNA) and ethidium homodimer (3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide; EthD-1). This protocol was originally described by Cooper & Yeung (1998) and has been adapted to boar spermatozoa in our laboratory. In brief, samples were incubated with EthD-1 (final concentration: 2.5 µg/mL) at 38 °C for 5 min in the dark. Following this step, samples were centrifuged at 2000 g for 30 sec and then resuspended with PBS containing 4 mg/mL bovine serum albumin (BSA) to remove the free dye. Thereafter, samples were again centrifuged at the aforementioned conditions and then fixed and permeabilised by adding 100 µL of ice-cold methanol (100%) for 30 sec. Methanol was removed by centrifugation

at 2000 g for 30 sec, and pellets were resuspended with 250 µL PBS. Following this step, 0.8 µL PNA-FITC (final concentration: 2.5 µm) was added, and samples were incubated at 15 °C in the dark for 15 min. Next, samples were washed twice with PBS at 2000 g for 30 sec and finally resuspended in PBS.

Following staining, samples were evaluated with the flow cytometry and the following four sperm populations were identified: (i) viable spermatozoa with an intact acrosome membrane (PNA-FITC+/EthD-1−); (ii) viable spermatozoa with a non-intact acrosome membrane (PNA-FITC-/EthD-1−); (iii) non-viable spermatozoa with an intact acrosome membrane (PNA-FITC+/EthD-1+); and (iv) non-viable spermatozoa with a non-intact acrosome membrane (PNA-FITC-/EthD-1+). Fluorescence of EthD-1 was detected through FL3, whereas that of PNA-FITC was detected through FL1.

Evaluation of sperm membrane lipid disorder

Lipid disorder of boar sperm membrane was evaluated by costaining with merocyanine-540 (M540) and YO-PRO-1, following a procedure modified from Rathi et al. (2001). Briefly, spermatozoa were stained with M540 (final concentration: 2.6 µm) and YO-PRO-1 (final concentration: 25 nm) and incubated at 38 °C for 10 min in the dark. Red fluorescence from M540 was collected through FL3, and green fluorescence from YO-PRO-1 was collected through FL1. The following four sperm populations were distinguished: (i) viable spermatozoa with low membrane lipid disorder (M540−/YO-PRO-1−); (ii) viable spermatozoa with high membrane lipid disorder (M540+/YO-PRO-1−); (iii) non-viable spermatozoa with low membrane lipid disorder (M540−/YO-PRO-1+); and (iv) non-viable spermatozoa with high membrane lipid disorder (M540+/YO-PRO-1+). In this test, data were not compensated.

Evaluation of intracellular levels of superoxides and peroxides

Intracellular superoxide (O2_●) and peroxide (H2O2) levels were determined using two different oxidation-sensitive fluorescent probes: hydroethidine (HE) and 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA). Following a procedure modified from Guthrie & Welch (2006), a simultaneous differentiation of viable from non-viable spermatozoa was performed using either YO-PRO-1 or PI.

In the case of superoxides, samples were stained with HE (final concentration: 4 µm) and YO-PRO®-1 (final concentration: 25 nm) and incubated at 15 °C for 40 min in the dark [17]. Hydroethidine is freely permeable to cells and is oxidised by O2−● to ethidium (E+) and other products. Fluorescence of ethidium (E+) was detected through FL3, and that of YO-PRO-1 was collected through FL1. Viable spermatozoa with high intracellular superoxide levels were positive for ethidium and negative for YO-PRO-1 (E+/YO-PRO-1−).

With regard to peroxides, spermatozoa were stained with H2DCFDA at a final concentration of 200 µm and PI at a final concentration of 10 µm, and incubated at 15 °C for 60 min in the dark. H2DCFDA is a cell-permeable, non-fluorescent probe that is intracellularly de-esterified and converted into highly fluorescent, 2′,7′-dichlorofluorescein (DCF+) upon oxidation (Guthrie & Welch, 2006). This DCF+ fluorescence was collected through FL1, whereas PI fluorescence was detected through FL3. Data were not compensated, and viable spermatozoa with high intracellular peroxide levels were positive for DCF and negative for PI (DCF+/PI-).

In both cases, unstained and single-stained samples were used for setting EV, FL1 and FL3 PMT voltages and data were not compensated.

Immunoblotting

Aliquots of 1 mL corresponding to each experimental point were centrifuged at 1000 g and 15 °C for 30 sec, and pellets were stored at −80 °C until the beginning of the assay. Pellets were resuspended and sonicated in 300 µL ice-cold lysis buffer containing 50 mm Tris-HCl, 1 mm EDTA, 10 mm EGTA, 25 mm dithiothreitol, 1.5% (v:v) Triton® X-100, 1 mm PMSF, 10 µg/mL leupeptin, 1 mm orthovanadate and 1 mm benzamidine (pH = 7.4). After 30 min on ice, the homogenised suspensions were centrifuged at 600 g and 4 °C for 20 min, and total protein content in supernatants was calculated through the Bradford method (Bradford, 1976) using a commercial kit (Bio-Rad Laboratories). Afterwards, samples were mixed with loading buffer (1 : 5; v:v) containing 250 mm Tris-HCl (pH = 6.8), 50 mm dithiothreitol, 10% (w:v) SDS, 0.5% (v:v) bromophenol blue and 50% (v:v) glycerol and stored at −20 °C until gel electrophoresis (SDS-PAGE; Laemmli, 1970).

Prepared samples were loaded onto 0.75-mm gels containing 10% acrylamide (w:v). After running the gels at constant voltage (180 V), proteins were transferred onto Immun-Blot® low-fluorescence polyvinylidene fluoride (PVDF) membranes (Bio-Rad) using the Trans-Blot® Turbo Transfer System with Trans-Blot® Turbo Midi Transfer Packs (Bio-Rad). Membranes were subsequently immersed for 60 min into blocking solution, consisting of Tris-buffered saline solution added with 5% (w:v) BSA and 0.1% (v:v) Tween-20. Thereafter, membranes were incubated with a mouse monoclonal PY20 antiphosphotyrosine antibody (ref. P4110; Sigma-Aldrich; dilution factor: 1 : 1000 (v:v) in blocking solution) at 4 °C for 8 h. Membranes were washed three times with blocking solution (5 min per wash) and then incubated at 15 °C for 1 h with a horseradish peroxidase (HRP)-conjugated, polyclonal rabbit anti-mouse antibody (Dako; Glostrup, Denmark) at a dilution of 1 : 5000 (v:v) in blocking solution. After washing membranes with blocking solution for six times (5 min per wash), membranes were incubated with chemiluminescent HRP substrate (ImmunoCruz Western Blotting Luminol Reagent; Santa Cruz Biotechnology®, Dallas, TX, USA) at 15 °C for 2 min, following manufacturer's instructions. Revealed images were analysed through imagej ver. 1.49 (National Institute of Health, Bethesda, MD, USA), and the intensity/densitometry of each band was quantified. Following this, membranes were stripped and then incubated with a mouse monoclonal anti-β-tubulin (ref. T5201; Sigma-Aldrich; 1 : 1000 (v:v) in blocking solution) and the same secondary HRP antibody. Images were also analysed through ImageJ. A total of seven semen samples were used for Western blot assays.

Evaluation of free cysteine residues in spermatozoa and tail extracts

Determination of free cysteine radicals in sperm head and tail extracts as an indirect measure of disrupted disulphide bridges within proteins was carried out following the protocol described in Flores et al. (2011). Briefly, samples were centrifuged at 600 g and 16 °C for 10 min and then resuspended in an ice-cold lysis buffer made up as follows: 50 mm Tris buffer, 150 mm NaCl, 1% (v:v) Non-idet, 0.5% (w:v) sodium deoxycholate, 1 mm benzamidine, 10 µg/mL leupeptin, 0.5 mm phenylmethylsulfonyl fluoride (PMSF) and 1 mm Na2VO4 (pH adjusted to 7.4). Samples were homogenised through sonication (12 pulses; Ikasonic U50 sonicator; Ika Labortechnick, Staufen, Germany), and obtained homogenates were centrifuged at 850 g and 4 °C for 20 min. After this centrifugation step, the supernatant mainly contained the sperm tails, whereas the pellet mainly contained the sperm heads. This was confirmed by separate evaluations through phase-contrast microscopy of both fractions, the pellets being previously resuspended with 300 µL Tris buffer. Indeed, percentages of tails in supernatants and heads in pellets were found to be above 90%, respectively (data not shown).

Levels of free cysteine radicals in both fractions (i.e. supernatants and Tris-solubilised pellets) were determined using the 2,2′-dithiodipyridine technique (2,2′-dipyridyl disulphide; Sigma-Aldrich) as

313 described in Brocklehurst et al. (1979). Briefly, 10 µL of the supernatant or resuspended pellet was
314 added with 990 mL of an aqueous solution containing 0.4 mm 2,2′-dithiodipyridine. Standard curves
315 were generated with 10-µL aliquots containing different concentrations of cysteine (Sigma-Aldrich;
316 from 0.1 to 5 mm), which were also added with 990 mL of 0.4 mm 2,2′-dithiodipyridine. Samples
317 were incubated at 37 °C for 60 min, and levels of free cysteine radicals were subsequently determined
318 through spectrophotometry at a wavelength of 340 nm. The results obtained were normalised through
319 a parallel determination of the total protein content by the Bradford method (Bradford, 1976), using
320 a commercial kit (Quick Start Bradford Protein Assay; Bio-Rad, Hercules, CA, USA). Five replicates
321 per sample and treatment were evaluated, and the corresponding mean ± standard error of the mean
322 (SEM) was calculated.

323 Distribution of lectins over sperm membrane

324 Four FITC-conjugated lectins were used as follows: Triticum vulgaris agglutinin (WGA), Solanum
325 lycopersicum lectin (STL), Pisum Sativum agglutinin (PSA) and Arachis Hypogaea agglutinin
326 (PNA). Semen samples were centrifuged at 1000 g and 15 °C for 30 sec, and the resultant pellets
327 were resuspended with 400 µL PBS containing 4% (w:v) paraformaldehyde. Fixation was conducted
328 at 4 °C in the dark for 2 h. Samples were subsequently spread onto poly-lysine (1% poly-lysine
329 solution in water; Sigma-Aldrich)-coated microscope slides and then left to dry. Samples were
330 permeabilised by incubation with 0.3% (v:v) Triton® X-100 in PBS (pH = 7.4) at 15 °C for 10 min.
331 Next, slides were washed three times with PBS and then blocked through incubation with PBS
332 containing 0.1% (v:v) Tween-20 and 5% (w:v) BSA at 15 °C for 60 min. After blocking, samples
333 were incubated at 15 °C in a humid chamber for 1 h with the corresponding lectin at the following
334 dilutions in PBS: 1 : 200 (w:v) for WGA and PSA, 1 : 300 (w:v) for PNA and 1 : 50 (w:v) for STL.
335 Slides were further washed three times with PBS (5 min each wash) and then mounted with antifading
336 medium Vectashield H-1000 (Vector Laboratories, Burlingame, CA, USA). After being covered by
337 coverslips, slides were compressed to eliminate any excess of liquid. Coverslips were finally sealed
338 with colourless nail polish, and slides were stored at 4 °C in the dark until observation. Negative
339 control experiments were performed omitting the lectin.

340 Samples were observed using a confocal laser scanning microscope (Leica TCS 4D; Leica
341 Lasertechnik, Heidelberg, Germany) at 63× magnification. The light source was an argon⁄krypton
342 laser. Successive confocal slices of images (image thickness: 0.5 µm) were integrated to create three-
343 dimensional images that were saved in TIFF format. Each lectin generated distinct staining patterns
344 that were examined in non-capacitated, capacitated and acrosome-exocytosed spermatozoa.

345 In vitro oocyte–sperm co-incubation and evaluation of sperm adhesiveness and 346 penetration ability

347 As previously mentioned, the current work was divided into two experiments. In the second
348 experiment, and following the results obtained in the first one, one melatonin treatment (1 µm) was
349 compared with the control. The sperm ability to bind the ZP and to penetrate in vitro matured oocytes
350 was evaluated after previous incubation with 1 µm melatonin, following a modified protocol from
351 Castillo-Martín et al. (2014).

352 Ovaries were obtained from a local slaughterhouse and were brought to the laboratory in a 0.9% (w:v)
353 NaCl solution containing 100 µg kanamycin sulphate per mL previously warmed at 37 °C. Oocyte–
354 cumulus cell complexes (COCs) were collected from follicles of 3–6 mm diameter and only those
355 showing at least two layers of cumulus cells and a homogeneous cytoplasm were selected. COCs
356 were washed twice with DPBS supplemented with 4 mg/mL polyvinyl alcohol (PVA) and then with
357 maturation medium, previously equilibrated at 38.5 °C and 5% CO2 in humidified air for at least 3 h.

Groups of 50 oocytes were cultured in 500 µL maturation medium for 22 h at 38.5 °C and 5% CO2 in humidified air. Thereafter, oocytes were transferred to fresh maturation medium without hormones or dibutyryl cAMP, and cultured for further 22 h. The maturation medium was NCSU-37 (Petters & Wells, 1993) supplemented with 0.57 mm cysteine, 1 mm dibutyryl cAMP, 5 µg/mL insulin, 50 µm β-mercaptoethanol, 10 IU/mL equine chorionic gonadotrophin (Folligon, Intervet International BV, Boxmeer), 10 IU/mL human chorionic gonadotrophin (Veterin Corion, Divasa Farmavic, Barcelona, Spain) and 10% (v:v) pig follicular fluid.

After maturation, oocytes were mechanically stripped of cumulus cells by gentle aspiration with a pipette. Denuded oocytes were washed with TALP medium, and groups of 25 oocytes were then transferred to each well of four-well Nunc multidishes (Nunc; Roskilde, Denmark) containing 250 µL TALP medium, previously equilibrated at 38.5 °C under 5% CO2 in humidified air. The composition of TALP medium was as follows: 114.06 mm NaCl, 3.2 mm KCl, 8 mm calcium lactate·5H2O, 0.5 mm MgCl2·6H2O, 0.35 mm NaH2PO4, 25.07 mm NaHCO3, 10 mL/L sodium lactate, 5 mm glucose, 2 mm caffeine, 1 g/L PVA, and 0.17 mm kanamycin sulphate supplemented with 3 mg/mL fatty acid-free BSA (FAF-BSA) and 1.1 mm sodium pyruvate (Rath et al., 1999).

Two hundred fifty microlitres of sperm suspensions from each treatment group was added to the fertilisation wells at a final concentration of 5 × 104 sperm/mL. Those spermatozoa had previously been incubated in CM at 38.5 °C and 5% CO2 in humidified air for 4 h, either in the presence or absence of 1 µm melatonin. Specifically, three treatments were set as follows: (i) control, which consisted of oocytes incubated with spermatozoa previously incubated in CM without melatonin; (ii) experimental treatment 1, which consisted of oocytes co-incubated with spermatozoa previously in vitro capacitated with CM added with 1 µm melatonin at 38.5 °C and 5% CO2 in humidified air for 4 h; and (iii) experimental treatment 2, which consisted of oocytes added with both spermatozoa (previously incubated in CM without melatonin) and melatonin to a final concentration of 1 µm. In all treatments, co-incubation of spermatozoa with in vitro matured oocytes was performed at 38.5 °C and 5% CO2 in humidified air for 1 h. Free, non-attached spermatozoa were removed by washing oocyte–sperm complexes with TALP medium, and 500 µL fresh TALP medium was subsequently added. Oocyte–sperm complexes were incubated at 38.5 °C and 5% CO2 in humidified air for further 7 h and subsequently prepared for nuclear staining. Following this, oocytes were gently aspirated with a pipette, washed with TALP medium and subsequently transferred to a new well containing 500 µL TALP. Oocyte–sperm complexes were maintained in this medium at 38.5 °C and 5% CO2 in humidified air for further 7 h and then collected to perform the following nuclear staining protocol.

Oocyte–sperm complexes were washed with warmed PBS and then fixed with 4% (w:v) paraformaldehyde in PBS at 38.5 °C for 30 min. After fixation, complexes were washed twice with PBS and subsequently stained with 1% (v:v) Hoechst® 33342 in PBS at 15 °C for 25 min. Oocyte–sperm complexes were then washed two times with PBS, mounted on glass slides and examined under a TCS 4D laser confocal scanning microscope (Leica Lasertechnik) at 63× magnification. The following parameters were evaluated: (i) spermatozoa bound to the ZP: number of nuclear spermatozoa attached to the ZP; (ii) total penetration rate: number of oocytes that showed evident signs of sperm penetration divided by the total number of sperm–oocytes complexes; lack of penetration consisted of sperm–oocytes complexes that showed a unique nucleus with or without apparent polar bodies; (iii) percentage of monospermy: number of oocytes showing the presence of two pronuclei, or one sperm head inside the oocyte with or without signs of decondensation, divided by the total number of sperm–oocytes complexes; and (iv): percentage of polyspermy: number of oocytes showing more than two pronuclei, or more than one sperm head inside the oocyte with or without signs of decondensation, divided by the total number of sperm–oocytes complexes.

Statistical analyses

Statistical analyses were performed using a statistical package (IBM SPSS for Windows version 21.0, IBM Corp; Chicago, IL, USA). Data are presented as mean ± standard error of the mean (SEM), and the level of significance was set at $p \leq 0.05$.

In the case of experiment 1, data were first tested for normality and homogeneity of variances through Shapiro–Wilk and Levene tests, respectively. When required, data (x) were transformed through arcsine square root ($\arcsin\sqrt{x}$) before a general mixed model (i.e. with repeated measures) was run. In this model, the intersubject factor was the treatment (i.e. composition of capacitation media), and the intrasubject factor was the incubation time (i.e. 0 h, 4 h, 4 h + 1 min, 4 h + 5 min, 4 h + 60 min). In all cases, each sperm functional parameter was the dependent variable, and pairwise comparisons were made with Sidak post hoc test. When no transformation remedied the normality, nonparametric procedures were conducted with raw data. Friedman's test and the Wilcoxon matched-pairs test were performed as nonparametric alternatives to repeated measures ANOVA.

With regard to experiment 2, the number of spermatozoa attached to oocyte ZP was checked for normality and homogeneity of variances as previously described, and compared through one-way ANOVA followed by post hoc Sidak's test. For the analysis of monospermy/polyspermy, a chi-square test ($\chi 2$) was used.

Results

Effects of melatonin on viability, acrosome exocytosis and capacitation-like changes in sperm membrane

As shown in Figure S1A, incubation of boar spermatozoa in CM for 4 h reduced their viability, which went from 80.4% ± 3.7% at 0 h to 67.9% ± 2.8% after 4 h of incubation. This decline was maintained after the addition of progesterone. While the addition of melatonin to CM at 0 h or 4 h did not significantly modify the observed drop in sperm viability, the extent of that decrease was higher when spermatozoa were incubated in NCM (Figure S1A,B).

Percentages of true acrosome exocytosis (PNA-FITC−/EthD-1−) were very low in cells incubated in CM during 4 h. The addition of progesterone at 4 h induced an increase in this percentage, which reached maximal values after 60 min of that addition (67.4% ± 2.3%; Figure S2A,B). This increase was not observed when spermatozoa were incubated in NCM. The addition of melatonin either at 0 h or at 4 h did not modify the pattern observed in spermatozoa incubated in CM (Figure S2A,B).

Incubation of boar spermatozoa in CM significantly ($p < 0.05$) increased the percentage of viable spermatozoa with high membrane lipid disorder (from 9.4% ± 2.6% at 0 h to 45.7% ± 4.6% at 4 h; Figure S3A,B). The subsequent addition of progesterone was associated with a progressive decrease in this percentage, which reached values of 34.6% ± 3.0% after 60 min of progesterone addition. The addition of melatonin either at 0 h or at 4 h did not change the dynamics observed in spermatozoa incubated in CM (i.e. positive control; Figure S3A,B).

Effects of melatonin on P32 tyrosine phosphorylation levels

As expected, incubation of boar spermatozoa in CM for 4 h induced a noticeable increase in tyrosine phosphorylation (pTyr) levels of P32 protein (from 100.0 arbitrary units at 0 h of incubation to 231.7 ± 14.3 arbitrary units after 4 h), which was roughly maintained after progesterone addition (Figures S4 and S5). Addition of melatonin at 0 h did not significantly modify that pattern (Figure S4). The addition of progesterone after 4 h of incubation in CM did not have any prominent effect on pTyr-

446 P32 levels. Only the treatment containing melatonin at 5 μm showed a slight decrease in pTyr-P32
447 values when compared to incubation in CM (60 min after progesterone addition; melatonin at 5 μm:
448 203.5 ± 7.4 arbitrary units vs. CM: 228.2 ± 7.6 arbitrary units; see Figure S5). Finally, the addition
449 of 5 μm melatonin at 4 h was found to decrease the intensity of tyrosine phosphorylation in P32 band
450 after 5 min and 60 min of progesterone addition (60 min after progesterone addition; melatonin at 5
451 μm: 206.8 ± 6.5 arbitrary units vs. CM: 226.1 ± 6.9 arbitrary units; see Figure S5).

452 Effects of melatonin on sperm motility

453 Total motility of spermatozoa incubated in CM significantly ($p < 0.05$) decreased throughout the
454 experiment, reaching minimal values of 27.0% ± 2.5% after 60 min of the addition of progesterone
455 (Fig. 1). Incubation of spermatozoa in NCM led to even worse motility values, with complete
456 immobilisation at the end of the experiment. The addition of melatonin at 0 h induced an immediate
457 decrease in total motility, which was more apparent at the highest melatonin concentration (0 h:
458 47.2% ± 3.0% in melatonin at 5 μm vs. 64.0% ± 3.9% in CM; Fig. 1A). This adverse effect on sperm
459 motility was observed throughout all the incubation period. Melatonin also decreased sperm motility
460 when added together with progesterone at 4 h, the treatments containing melatonin at 1 and 5 μm
461 showing values near to complete immobility after 60 min of progesterone addition (Fig. 1B).

462 Regarding kinetic parameters, spermatozoa incubated in CM for 4 h showed significant ($p < 0.05$)
463 increases in several parameters, including VCL, VAP and ALH (as an example, VAP at 0 h of
464 incubation in CM: 65.7 μm/sec ± 2.1 μm/sec vs. VAP after 4 h of incubation in CM: 72.9 μm/sec ±
465 2.9 μm/sec; Table 1). However, the addition of melatonin at 0 h significantly ($p < 0.05$) decreased
466 VAP values (after 4 h of incubation; 57.9 μm/sec ± 1.7 μm/sec in the treatment containing 0.5 μm
467 melatonin vs. 72.9 μm/sec ± 2.9 μm/sec in CM; Table 1). When melatonin was added together with
468 progesterone at 4 h, there was an immediate decrease in VAP (1 min after progesterone addition: 40.9
469 μm/sec ± 2.0 μm/sec in the treatment containing 1 μm melatonin vs. 59.8 μm/sec ± 2.7 μm/sec in
470 CM; Table 1), LIN (1 min after progesterone addition: 25.7% ± 1.6% in the treatment containing 1
471 μm melatonin vs. 39.9% ± 1.7% in CM; Table 2) and STR (1 min after progesterone addition: 58.1%
472 ± 1.8% in the treatment containing 1 μm melatonin vs. 67.7% ± 2.8% in CM; Table 2). Melatonin-
473 induced decreases of both VAP and STR were recovered after 5 min and 60 min of the addition of
474 progesterone and melatonin at 0.5 and 1 μm (VAP after 60 min of the addition of progesterone and
475 melatonin at 1 μm: 50.1 μm/sec ± 3.1 μm/sec vs. 56.8 μm/sec ± 3.3 μm/sec in CM; STR after 60 min
476 of the addition of progesterone and melatonin at 1 μm: 77.1% ± 4.4% vs. 76.2% ± 3.3% in CM;
477 Tables 1 and 2). In contrast, LIN was only recovered after 60 min of the addition of progesterone and
478 melatonin at the same concentrations (LIN after 60 min of the addition of progesterone and melatonin
479 at 1 μm: 32.7% ± 1.5% vs. 37.1% ± 2.0% in CM; Table 2).

480 Effects of melatonin on sperm agglutination

481 Incubation of spermatozoa in CM increased their degree agglutination, which was 60.9% ± 7.5% at
482 4 h (Fig. 2). Agglutinations were of medium size (Figure S6D,E), and about 45% of agglutinated
483 spermatozoa showed appreciable tail beating at 4 h of incubation (Fig. 3). Although the percentage
484 of agglutinated spermatozoa did not vary after the addition of progesterone (Fig. 2, Figure S6F), the
485 percentage of agglutinated spermatozoa with appreciable tail beating showed a transient increase
486 upon progesterone addition and then started to decrease, reaching a value of 17.3% ± 2.6% at the end
487 of the experiment (Fig. 3). In contrast to the aforementioned, spermatozoa incubated in NCM did
488 show low percentages of agglutinated spermatozoa (Fig. 2, Figure S6A–C). The addition of melatonin
489 at 0 h induced an immediate and significant ($p < 0.05$) increase in the percentage of agglutinated
490 spermatozoa (5 μm melatonin: 56.2% ± 6.4% vs. CM: 26.1% ± 3.8%; Fig. 2A and Figure S6G). This
491 increase continued and reached values of about 80–85% at 4 h, when more than a hundred

spermatozoa were observed in a single agglutination (Figure S6H). Similar results were found throughout the remaining experimental period (Fig. 2A, Figure S6I).

Regarding the percentage of agglutinated spermatozoa with appreciable tail beating, melatonin induced a significant ($p < 0.05$) decrease in all the tested concentrations, reaching minimal values at 4 h (melatonin µm: 12.8% ± 1.9% vs. CM: 43.2% ± 3.2%; Fig. 3A). After progesterone addition, a similar decreasing pattern was observed. Finally, the addition of both 1 and 5 µm melatonin at 4 h did counteract the decreases in the percentages of agglutination and of agglutinated spermatozoa with tail beating observed in control samples 60 min after progesterone addition (Fig. 3; Figure S6J).

Effects of melatonin on intracellular ROS levels

Incubation of boar spermatozoa in CM induced a slight, but significant ($p < 0.05$) increase in the percentage of viable spermatozoa with high intracellular H2O2 levels, which went from 1.6% ± 0.2% at 0 h to 5.8% ± 1.3% at 4 h (Fig. 5A,B). This was in contrast with sperm cells incubated in NCM in which the extent of that increase was higher (9.4% ± 2.5% at 4 h; Fig. 4). The subsequent addition of progesterone did not significantly modify the percentage of high-H2O2 cells in spermatozoa incubated in CM, whereas those incubated in NCM showed a slight and gradual increase, reaching values of 13.6% ± 2.9% after 60 min of progesterone addition (Fig. 4).

The addition of melatonin at 0 h did not significantly affect the pattern observed in CM, except in the case of melatonin 5 µm, where there was a significant ($p < 0.05$) decrease in the percentage of cells with high H2O2 levels after 1 min of progesterone addition that was not further recovered (Fig. 4A). The addition of melatonin at 1 or 5 µm at 4 h showed a significant ($p < 0.05$) decrease in this percentage at 1 min post-progesterone addition (Fig. 4B).

Percentages of viable spermatozoa with high intracellular O2−● levels slightly decreased throughout incubation time and went from 1.4% ± 0.2% at 0 h to 6.2% ± 1.7% at 4 h (Fig. 5A,B). Subsequent addition of progesterone did not have a remarkable effect on this parameter, and a slight increase was seen after 60 min of progesterone addition (9.0% ± 2.8%; Fig. 5A,B). In the case of incubation in NCM, the values were significantly higher, reaching values of 19.8% ± 4.1% after 60 min of progesterone addition. The addition of melatonin at any of the tested concentrations either at 0 h or at 4 h did not differ from spermatozoa incubated in CM (Fig. 5A,B).

Effects of melatonin on the free cysteine residues in both head and tail sperm extracts

Incubation of boar spermatozoa in CM induced a progressive increase in the free cysteine levels from head extracts which went from 3.9 nmol/g protein ± 0.3 nmol/g protein at 0 h to 17.2 nmol/g protein ± 2.3 nmol/g protein at 4 h (Fig. 6A,B). This increase was not observed in sperm cells incubated in NCM. Subsequent addition of progesterone to spermatozoa incubated in CM did not increase these levels, and there was a slight gradual decrease with values of 11.3 nmol/g protein ± 1.7 nmol/g protein after 60 min of progesterone addition (Fig. 6A,B).

Addition of melatonin at 0 h had a dramatic effect on free cysteine levels from head extracts, as almost abolished the increase observed in CM (Fig. 6A). This effect was maintained after the addition of progesterone. When melatonin was added at 4 h, a similar decreasing effect on free cysteine levels of sperm head extracts was immediately observed (i.e. 1 min after melatonin and progesterone addition), especially at the highest concentrations.

533 Free cysteine levels of sperm tail extracts also increased in spermatozoa incubated in CM. Values
534 went from 3.7 nmol/g protein ± 0.6 nmol/g protein at 0 h to 9.3 nmol/g protein ± 1.9 nmol/g protein
535 at 4 h (Fig. 7A,B). These values were roughly maintained after the addition of progesterone. The
536 addition of melatonin at 0 h almost abolished that increase at any concentration tested (Fig. 7A). On
537 the contrary, the addition of melatonin at 4 h had no clear effect on this parameter before 60 min after
538 progesterone addition, when free cysteine levels of sperm tail extracts were found to increase in a
539 melatonin dose-dependent manner (5 μm: 12.8 nmol/g protein ± 2.4 nmol/g protein vs. CM: 8.9
540 nmol/g protein ± 1.7 nmol/g; Fig. 7A).

541 Effects of melatonin on distribution of lectins over sperm membrane

542 At the beginning of incubation in CM, WGA signal was located at the sperm head and the whole tail,
543 although the maximal intensity of the signal was observed at the acrosomal edge (Figure S7).
544 Spermatozoa incubated in NCM showed a similar staining, but the acrosomal signal was much less
545 intense. After 4 h of incubation in CM, the intensity of the acrosome-located signal increased and
546 uniformly distributed throughout the entire acrosome (Figure S7). These changes were not detected
547 in spermatozoa incubated in NCM for 4 h. The subsequent addition of progesterone to CM induced
548 further modifications in the acrosome signal of WGA. In effect, although small changes were
549 observed after 1 min of adding progesterone, with some sperm cells losing the inner lectin signal and
550 others showing an irregular acrosome marking, the progesterone-induced changes were much more
551 apparent after 5 min, when a high number of sperm cells showed a diffuse inner or irregular acrosome
552 signal (Figure S7). These patterns were also observed after 60 min of progesterone addition. Addition
553 of melatonin at 0 h induced changes in the WGA-staining pattern. At 4 h, most of the spermatozoa
554 incubated with melatonin showed much more intense acrosome signal than control spermatozoa
555 (CM), but there were also sperm cells that showed no lectin signal in the post-acrosomal area and
556 even spermatozoa with no signal over the head (Figure S7). The addition of progesterone to
557 spermatozoa incubated for 4 h in treatments containing melatonin did not induce immediate changes
558 in WGA distribution. However, the acrosome staining in spermatozoa incubated with melatonin was
559 more intense than that observed in control spermatozoa incubated in CM after 60 min of incubation
560 (Figure S7). In contrast, the addition of 1 μm melatonin at 4 h did not change WGA distribution when
561 compared to control spermatozoa incubated in CM (Figure S7).

562 Regarding STL, it was mainly found in sperm head and midpiece (in some cells, STL was observed
563 in the entire tail) at 0 h, with a more intense signal in the acrosome area (Figure S8). Following
564 incubation in CM for 4 h, two different STL-staining patterns were observed. Whereas one pattern
565 consisted of an intense and uniform signal throughout the entire acrosome area, STL staining in the
566 other was mainly restricted to the acrosomal edge (Figure S8). In both patterns, STL signal in the
567 midpiece was much decreased or totally absent. The addition of progesterone at 4 h decreased the
568 intensity of acrosome signal in practically all sperm cells (which was already apparent after 1 min of
569 progesterone addition) but increased that of the tail (Figure S8). Addition of 1 μm melatonin at 0 h
570 exhibited similar STL-staining patterns to the control (Figure S8) and there was a clear loss of STL
571 signal in the acrosome following progesterone addition, the STL-staining being restricted to the
572 acrosome edge. While the addition of 1 μm melatonin at 4 h did not clearly affect the STL pattern
573 observed in spermatozoa incubated in CM, a high proportion of spermatozoa showed a clearly intense
574 acrosome signal after 1 min of the addition of melatonin and progesterone, which differed from
575 spermatozoa incubated in CM (Figure S8).

576 At 0 h, PSA staining was observed in the whole cell (spermatozoa incubated in CM), although the
577 most intense marking was detected in the entire acrosomal area. In the case of spermatozoa incubated
578 in NCM, the acrosomal signal was much less intense and restricted to the acrosome edge (Figure S9).
579 Incubation in CM for 4 h induced an increase in the PSA-staining of the sperm head and tail, despite

580 the post-acrosomal region being devoid of PSA signal. Subsequent addition of progesterone induced
581 a rapid loss of PSA signal in the acrosome, which was evident in a high percentage of spermatozoa
582 after 1 min of the addition of the hormone (Figure S9). In spite of this, there were other spermatozoa
583 that showed an intense acrosome signal. The addition of 1 μm melatonin either at 0 h or at 4 h did not
584 have a clear effect on PSA localisation during IVC and IVAE (Figure S9).

585 Regarding the localisation of PNA, it was exclusively found at the whole acrosome surface and there
586 were no changes after 4 h of incubation either in CM or in NCM (Figure S10). As expected, the
587 addition of progesterone at 4 h increased the proportion of spermatozoa with less intensity of PNA
588 marking and faint staining restricted to the acrosome edge (Figure S10). These changes were very
589 rapid, as they were already observed after 1 min of progesterone addition. Addition of melatonin
590 either at 0 h or at 4 h showed no differences when compared to spermatozoa incubated in CM (Figure
591 S10).

592 Effects of melatonin on the ability of in vitro capacitated boar spermatozoa to adhere and penetrate
593 pig oocytes

594 As shown in Table 4, the number of spermatozoa attached to the ZP (78.4 ± 1.8), the total penetration
595 rate (90.6%) and the percentage of monospermy (69.8%) were higher in the control group (CM) than
596 in the other treatments. Previous incubation of spermatozoa with 1 μm melatonin significantly ($p <$
597 0.05) decreased the number of spermatozoa adhered to the ZP (68.2 ± 2.7 vs. 78.4 ± 1.8 in CM).
598 Furthermore, incubation with melatonin at 1 μm significantly ($p < 0.05$) decreased the proportion of
599 polyspermic oocytes (14.5% vs. 20.8% in CM). The addition of 1 μm melatonin after in vitro
600 capacitation for 4 h had no effect on polyspermy, but increased the number of spermatozoa attached
601 to the ZP (88.9 ± 1.7 vs. 78.4 ± 1.8 in CM; Table 4).

602 Discussion

603 The results shown herein suggest that one of the most important effects of melatonin during the
604 achievement of boar spermatozoa IVC is the increase in cell adhesiveness. The increase in sperm
605 adhesiveness would influence important aspects of boar sperm capacitation such as sperm motility
606 and sperm–zona pellucida interaction through the activation of sperm agglutination, as the results
607 suggested (Fig. 2 and Tables 1-3). Additionally, melatonin did not affect several of the most important
608 capacitation markers, such as membrane lipid disorder, tyrosine phosphorylation levels of P32 and
609 the ability to reach acrosome exocytosis after progesterone stimulation. This lack of additional effects
610 could suggest that the melatonin action of IVC is mainly focused on motility and/or sperm
611 adhesiveness. Regarding the relationship between sperm motility and agglutination, it is worth noting
612 that IVC in species such as the monkey, cattle, sheep and pig leads to an increase in the percentage
613 of agglutinated spermatozoa (Boatman & Bavister, 1984; Ehrenwald et al., 1990; Funahashi & Day,
614 1993; Lefebvre & Suarez, 1996; Leahy et al., 2016). In fact, sperm agglutination does not only result
615 from IVC, but also from other factors, such as the presence of antibodies (Yakirevich & Naota, 1999)
616 or in response to cell degeneration (Harayama et al., 1998). Furthermore, several components of
617 capacitation media, such as heparin in cattle, BSA in horse, and bicarbonate and calcium in pigs,
618 monkeys and cattle increase sperm agglutination (Lindahl & Sjöblom, 1981; Boatman & Bavister,
619 1984; Ehrenwald et al., 1990; Funahashi & Day, 1993; Lefebvre & Suarez, 1996; Harayama et al.,
620 1998; Harayama & Kato, 2002). Therefore, in our experimental conditions, melatonin could have
621 enhanced the agglutination-promoting effect of CM components. This hypothesis could explain why
622 the addition of melatonin at concentrations lower than those assayed in this work (100 pM) does not
623 agglutinate ram spermatozoa (Casao et al., 2009). Based on CM composition, one could suggest that
624 bicarbonate is the agglutinating factor whose action is potentiated by the addition of melatonin. This
625 hypothesis is based on previous works from our laboratory, in which IVC of boar spermatozoa was

626 achieved in a medium without bicarbonate and not much agglutination was observed (Ramió et al.,
627 2008; Ramió-Lluch et al., 2011; Ramió-Lluch et al., 2014). While we cannot determine the exact
628 mechanism through which melatonin could enhance sperm agglutination, our results suggest it is
629 unlikely to be related to changes in the glycocalix composition of membrane surface, as lectin-binding
630 assays did not show apparent changes (Figures S7–S10). Therefore, we could propose two
631 explanations. The first one would be related to a cAMP-mediated mechanism via the activation of the
632 bicarbonate-sensitive adenylyl cyclase and PKA (Harayama & Kato, 2002). Another possible
633 mechanism would involve the maintenance of disulphide bonds, as melatonin induced a clear
634 decrease in the intracellular free cysteine levels of boar spermatozoa. In this respect, it is worth noting
635 that penicillamine has a potent action against agglutination in ram spermatozoa subjected to IVC
636 (Leahy et al., 2016), and one of the mechanisms through which it exerts that effect is linked to its
637 direct action on disulphide radicals, which are converted into sulfhydryl groups (Talevi et al., 2007;
638 Gualtieri et al., 2009). These data would be in concordance with the results obtained in this study,
639 suggesting that the effects of melatonin on sperm agglutination and free cysteine levels could be
640 linked. However, more work is needed to further elucidate this point.

641 The results observed following sperm–oocyte co-incubation could also be a consequence of
642 melatonin action on sperm agglutination rather than on other capacitation-related changes such as the
643 ability to trigger acrosome exocytosis following the appropriate stimuli, namely progesterone. Thus,
644 the reduction in the number of spermatozoa adhered to the ZP when they were previously capacitated
645 in the presence of melatonin could be a consequence of an increased degree of sperm agglutination,
646 thereby lowering the number of free spermatozoa able to adhere the ZP. Furthermore, as shown in
647 Table 4, the addition of melatonin after IVC significantly increased the number of spermatozoa bound
648 to the ZP. The combined analysis of these results suggests that the melatonin-induced agglutinating
649 effect in IVC conditions could be linked to an unspecific, increased adherence ability of boar
650 spermatozoa to other sperm cells or the oocyte. In turn, the observed decrease in polyspermy in
651 spermatozoa capacitated in the presence of melatonin could result from the decrease in the number
652 of adhered spermatozoa.

653 Regarding the effects of melatonin on the proportions of boar spermatozoa with high intracellular
654 ROS levels, results displayed in Figs 4 and 5 indicate that the antioxidant action of melatonin does
655 not seem to play a prominent role in the effects observed during IVC and IVAE and, specifically, in
656 the increase in sperm agglutination. While this conclusion could be surprising at first glance, one
657 should note that boar spermatozoa are characterised by a very low ROS production rate, even when
658 subjected to treatments that, such as freeze-thawing, induce the generation of high intracellular ROS
659 in other species (Bilodeau et al., 2000; Guthrie & Welch, 2006; Yeste et al., 2013, 2015a). This feature
660 is different from other species, such as the horse, in which ROS production is more intense (see Gibb
661 & Aitken, 2016; as a review). As a consequence, in species such as equine, bovine and human,
662 melatonin has a clear antioxidant effect and affects membrane lipid peroxidation and ROS levels
663 (Gadella et al., 2008; Rao & Gangadharan, 2008; Du Plessis et al., 2010; Jang et al., 2010; Da Silva
664 et al., 2011; Najafi et al., 2018). In fact, the antioxidant effect of melatonin in species such as the
665 horse is not only related to a direct action on the oxidative potential but also to intracellular Na+
666 concentrations, which also affect the overall redox status (Ortega Ferrusola et al., 2017). Taking all
667 of these data into consideration, an expected antioxidant effect of melatonin on boar spermatozoa
668 should be very subtle, if detectable. A possible reason to explain why boar spermatozoa do not
669 accumulate high ROS levels, and thus why melatonin would exert a slight action on this parameter,
670 could be linked to a species-specific mitochondria function. Previous reports have suggested that,
671 despite being important to maintain crucial sperm functions, such as motility, mitochondrial-
672 produced energy is low in boar spermatozoa (Rodríguez-Gil & Bonet, 2016). Indeed, O2 consumption
673 rate and intracellular ATP levels have been reported to be low during IVC and subsequent IVAE
674 (Ramió-Lluch et al., 2014). In addition to this, induction of IVC in the presence of oligomycin A, a

675 specific inhibitor of the mitochondrial ATP synthase, does not decrease either O2 consumption rates
676 or ATP levels (Ramió-Lluch et al., 2014), which suggests that boar sperm mitochondria are in an
677 uncoupled status throughout most of their lifespan. A consequence of this uncoupled status is the low
678 rate of ROS production due to substimulation of the electronic chain, which is the most important
679 ROS-synthesising point (Rodríguez-Gil & Bonet, 2016). Interestingly, progesterone-induced
680 acrosome exocytosis is concomitant with a sudden and intense peak in both O2 consumption rate and
681 intracellular ATP levels (Balis et al., 1999; Gualtieri et al., 2009), which suggests that mitochondria
682 are coupled at this moment. Following this rationale, mitochondrial coupling upon progesterone
683 addition would be associated with a transient increase in ROS generation, especially that of peroxides
684 (Tait & Green, 2012). This hypothesis matches with our observations, as the addition of melatonin
685 together with that of progesterone was found to decrease the proportions of viable spermatozoa with
686 high peroxide levels (see Fig. 4A). However, the fact that such a decrease did not modify the
687 percentage of acrosome-exocytosed spermatozoa induced by progesterone suggests that peroxide
688 levels do not play a vital role for acrosome reaction in boar spermatozoa, at least under our in vitro
689 conditions.

690 As indicated when discussing the effects on sperm agglutination and adhesiveness, melatonin
691 decreased the intracellular free cysteine levels in both sperm head and tail extracts (see Figs 6 and 7).
692 Free cysteine levels are an indirect marker of the number of disrupted disulphide bonds, as despite
693 not all free cysteine radicals resulting from the breakage of disulphide bonds, a significant percentage
694 has this origin (Yeste et al., 2013, 2014). Our results also indicated that the melatonin-induced
695 decrease in free cysteine residues of sperm head has no impact on achieving the IVC and subsequent
696 progesterone-induced IVAE. Another issue is the observed increase in free cysteine residues in sperm
697 tail extracts during IVC and the melatonin-counteracting effect of that increase. Disulphide bonds are
698 important for the maintenance of a proper sperm flagellum structure (Ijiri et al., 2014). Specifically,
699 disulphide bonds are crucial for a protein associated with the outer dense fibre 1 (ODF1; Cabrillana
700 et al., 2011). Thus, one could suggest that changes in free cysteine residues of sperm tail would affect
701 the structure of the flagellum which could, in turn, induce subtle changes in sperm motion. Related
702 to this, cleavage of disulphide bonds in mouse hexokinase-I isozyme 1 (HK1) is related to the
703 initiation of sperm motility (Nakamura et al., 2008). Thus, it could be hypothesised that changes in
704 the number of disrupted disulphide bonds along the flagellum, specifically in flagellum-bond-related
705 proteins such as sperm hexokinase-1 (Cabrillana et al., 2011, 2016), could also be related to the
706 changes in motion parameters observed during IVC. Herein, melatonin at 0.5 and 1 μm was found to
707 abolish the IVC-linked changes in kinetic parameters such as VCL, VAP and ALH (see Tables 1-3).
708 While this effect could also be due to other mechanisms, such as sperm agglutination, the possibility
709 that melatonin affects sperm motility through regulating the number of disrupted disulphide bonds
710 should not be dismissed.

711 It is worth noting that the addition of melatonin together with progesterone after 4 h of IVC has almost
712 no effects on subsequent acrosome exocytosis or on the sperm ability to adhere and penetrate oocytes
713 in vitro. Taking into account that the main difference of spermatozoa before and after their incubation
714 for 4 h in CM is the achievement of a feasible capacitation status, these results suggest that the
715 observed effects of melatonin on parameters such as the adhesiveness and free cysteine levels of the
716 tail are linked to the precise sperm status. Thus, and as indicated by membrane lipid disorder, sperm
717 motility and sensitivity to progesterone, capacitated differs from uncapacitated spermatozoa in their
718 response to melatonin. At this moment, it is not possible to ascertain which the basis for these
719 differences in melatonin action is. Further research focusing on the function of specific melatonin
720 receptors MT1 and MT2, which are present in boar spermatozoa (González-Arto et al., 2016), is
721 warranted.

In conclusion, melatonin modulates the achievement of IVC and subsequent progesterone-induced IVAE in boar spermatozoa via mechanism/s involved in the control of sperm motion through changes in the number of tail disulphide bridges, adhesiveness and further oocyte penetration ability. In addition, prevention of the IVC-induced increase in the disulphide bonds of sperm head proteins mediated by melatonin could also be relevant. Remarkably, melatonin effects on IVC/IVAE in boar spermatozoa do not seem to be related to a direct action on intracellular ROS levels, thus opening up alternative, perhaps receptor-mediated, pathways to explain the effects of this hormone upon sperm capacitation.

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Conflict of Interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported herein.

Authors' Contributions

M.R. and R.B. carried out the majority of the experimental work, collaborated in the design of the experiments and wrote the manuscript. A.Pl., M.S., A.Pe. and T.R. collaborated with M.R. and R.B. in performing laboratory work. J.M.F.N. conducted confocal analysis of lectin location. T.M.B., A.C. and J.A.C.P. collaborated in designing the experiments and critically revised the manuscript. S.B. and M.C.M. helped conduct flow cytometry analyses and IVF experiments. As joint senior author, M.Y. was involved in flow cytometry analyses and IVF experiments, designed the experiments, analysed the data, wrote the manuscript and gave his final approval. J.E.R.G. designed the experiments, analysed the data, wrote the manuscript and gave his final approval.

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REFERENCES

Ashrafi I, Kohram H & Ardabili FF. (2013) Antioxidative effects of melatonin on kinetics, microscopic and oxidative parameters of cryopreserved bull spermatozoa. Anim Reprod Sci 139, 25–30.

Awda BJ, Mackenzie-Bell M & Buhr MM. (2009) Reactive oxygen species and boar sperm function. Biol Reprod 81, 553–561.

Balis UJ, Behnia K, Dwarakanath B & Bathia SN. (1999) Oxygen consumption characteristics of porcine hepatocytes. Metab Eng 1, 49–62.

757 Bilodeau JF, Chatterjee S, Sirard NA & Gagnon C. (2000) Levels of antioxidant defenses are
758 decreased in bovine spermatozoa after a cycle of freezing and thawing. Mol Reprod Dev 55, 282–
759 288.

760 Boatman DE & Bavister BD. (1984) Stimulation of rhesus monkey sperm capacitation by cyclic
761 nucleotide mediators. J Reprod Fertil 71, 357–366.

762 Bradford MM. (1976) Rapid and sensitive method for quantitation of microgram quantities of protein
763 utilizing principle of protein-dye binding. Anal Biochem 72, 248–254.

764 Bravo MM, Aparicio IM, Garcıa-Herreros M, Gil MC, Pena FJ & Garcıa-Marın LJ. (2005) Changes
765 in tyrosine phosphorylation associated with true capacitation and capacitation-like state in boar
766 spermatozoa. Mol Reprod Dev 71, 88–96.

767 Brocklehurst K, Stuchbury T & Malthouse JPG. (1979) Reactivities of neutral and cationic forms of
768 2,20-dipyridyl disulfide towards thiolate anions - detection of differences between the active-centers
769 of actinidin, papain and ficin by a 3-protonic-state reactivity probe. Biochem J 183, 233–238.

770 Cabrillana ME, Monclus MA, Saez Lancellotti TE, Boarelli PV, Clementi MA, Vincenti AE, Yunes
771 RFM & Fornes MW. (2011) Characterization of flagellar cysteine-rich sperm proteins involved in
772 motility, by the combination of cellular fractionation, fluorescence detection, and mass spectrometry
773 analysis. Cytoskeleton 68, 491–500.

774 Cabrillana ME, Uribe P, Villegas JV, Alvarez J, Sanchez R & Fornes MW. (2016) Thiol oxidation
775 by nitrosative stress: cellular localization in human spermatozoa. Syst Biol Reprod Med 62, 325–334.

776 Casao A, Mendoza N, Perez-Pe R, Grasa P, Abecia JA, Forcada F, Cebrian-Perez JA & Muino-
777 Blanco T. (2009) Melatonin prevents capacitation and apoptotic-like changes of ram spermatozoa
778 and increases fertility rate. J Pineal Res 48, 39–46.

779 Casao A, Cebrian I, Asumpcao ME, Perez-Pe R, Abecia JA, Forcada F, Cebrian-Perez JA & Muino-
780 Blanco T. (2010) Seasonal variations of melatonin in ram seminal plasma are correlated to those of
781 testosterone and antioxidant enzymes. Reprod Biol Endocrinol 8, 59.

782 Castillo-Martın M, Bonet S, Morato R & Yeste M. (2014) Comparative effects of adding b-
783 mercaptoethanol or L-ascorbic acid to culture or vitrification-warming media on IVF porcine
784 embryos. Reprod Fertil Dev 26, 875–882.

785 Cebrian-Perez JA, Casao A, Gonzalez-Arto M, dos Santos Hamilton TR, Perez-Pe R & Muino-
786 Blanco T. (2014) Melatonin in sperm biology: breaking paradigms. Reprod Domest Anim 49, 1–21.

787 Cooper TG & Yeung CH. (1998) A flow cytometric technique using peanut agglutinin for evaluating
788 acrosomal loss from human spermatozoa. J Androl 19, 542–550.

789 Da Silva CM, Macıas-Garcıa B, Miro-Moran A, Gonzalez-Fernandez L, Morillo-Rodriguez A,
790 Ortega-Ferrusola C, Gallardo-Bolanos JM, Stilwell G, Tapia JA & Pena FJ. (2011) Melatonin reduces
791 lipid peroxidation and apoptotic-like changes in stallion spermatozoa. J Pineal Res 51, 172–179.

792 Du Plessis SS, Hagenaar K & Lampiao F. (2010) The in vitro effects of melatonin on human sperm
793 function and its scavenging activities on NO and ROS. Andrologia 42, 112–116.

794 Ehrenwald E, Foote RH & Parks JE. (1990) Bovine oviductal fluid components and their potential
795 role in sperm cholesterol efflux. Mol Reprod Develop 25, 195–204.

796 Espino J, Ortiz A, Bejarano I, Lozano GM, Monllor F, Garcıa JF, Rodrıguez AB & Pariente JA.
797 (2011) Melatonin protects human spermatozoa from apoptosis via melatonin receptor– and
798 extracellular signal–regulated kinase-mediated pathways. Fertil Steril 95, 2290–2296.

799 Flores E, Ramio-Lluch L, Bucci D, Fernandez-Novell JM, Pena A & Rodriguez-Gil JE. (2011)
800 Freezing-thawing induces alterations in histone H1-DNA binding and the breaking of protein-DNA
801 disulfide bonds in boar sperm. Theriogenology 76, 1450–1464.

802 Funahashi H & Day BN. (1993) Effects of follicular fluid at fertilization in vitro on sperm penetration
803 in pig oocytes. J Reprod Fertil 99, 97–103.

804 Gadella BM, Tsai PS, Boerke A & Brewis IA. (2008) Sperm head membrane reorganisation during
805 capacitation. Int J Dev Biol 52, 473–480.

806 Garner DL & Johnson LA. (1995) Viability assessment of mammalian sperm using sybr-14 and
807 propidium iodide. Biol Reprod 53, 276–284.

808 Gibb Z & Aitken RJ (2016) The impact of sperm metabolism during in vitro storage: the stallion as
809 a model. Biomed Res Int 2016, 1–8.

810 Gonzalez-Arto M, Carrillo AV, Martınez-Pastor F, Fernandez-Alegre E, Roca J, Miro J, Rigau T,
811 Rodrıguez-Gil JE, Perez-Pe R, Muino-Blanco T, Cebrian-Perez JA & Casao A. (2016) Melatonin
812 receptors MT1 and MT2 are expressed in spermatozoa from several seasonal and nonseasonal breeder
813 species. Theriogenology 86, 1958–1968.

814 Gualtieri R, Mollo V, Duma G & Talevi R. (2009) Redox control of surface protein sulphhydryls in
815 bovine spermatozoa reversibly modulates sperm adhesion to the oviductal epithelium and
816 capacitation. Reproduction 138, 33–43.

817 Guthrie HD & Welch GR. (2006) Determination of intracellular reactive oxygen species and high
818 mitochondrial membrane potential in Percoll-treated viable boar sperm using fluorescence-activated
819 flow cytometry. J Anim Sci 84, 2089–2100.

820 Harayama H & Kato S. (2002) Relationship between bicarbonate and cyclic nucleotide in the
821 promoting effects on head-to-head agglutination in boar spermatozoa. Asian J Androl 4, 87–96.

822 Harayama H, Miyake M, Shidara O, Iwamoto E & Kato S. (1998) Effects of calcium and bicarbonate
823 on head-to-head agglutination in ejaculated boar spermatozoa. Reprod Fertil Dev 10, 445–450.

824 Ijiri TW, Vadnais ML, Huang AP, Lin AM, Levin LR, Buck J & Gerton GL. (2014) Thiol changes
825 during epididymal maturation: a link to flagellar angulation in mouse spermatozoa? Andrology 2, 65–
826 75.

827 Jang HY, Kim YH, Kim BW, Park IC, Cheong HT, Kim JT, Park CK, Kong HS, Lee HK & Yang
828 BK. (2010) Ameliorative effects of melatonin against hydrogen peroxide induced oxidative stress on
829 boar sperm characteristics and subsequent in vitro embryo development. Reprod Domest Anim 45,
830 943–950.

Jimenez I, Gonzalez-Marquez H, Ortiz R, Herrera JA, Garcia A, Betancourt M & Fierro R. (2003) Changes in the distribution of lectin receptors during capacitation and acrosome reaction in boar spermatozoa. Theriogenology 59, 1171–1180.

Laemmli UK. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Leahy T, Rickard JP, Aitken RJ & de Graaf SP. (2016) Penicillamine prevents ram sperm agglutination in media that support capacitation. Reproduction 151, 167–177.

Lee JA, Spidlen J, Boyce K, Cai J, Crosbie N, Dalphin M, Furlong J, Gasparetto M, Goldberg M, Goralczyk EM, Hyun B, Jansen K, Kollmann T, Kong M, Leif R, McWeeney S, Moloshok TD, Moore W, Nolan G, Nolan J, Nikolich-Zugich J, Parrish D, Purcell B, Qian Y, Selvaraj B, Smith C, Tchuvatkina O, Wertheimer A, Wilkinson P, Wilson C, Wood J, Zigon R, International Society for Advancement of Cytometry Data Standards Task Force, Scheuermann RH & Brinkman RR. (2008) MIFlowCyt: the minimum information about a flow cytometry experiment. Cytometry 73A, 926–930.

Lefebvre R & Suarez SS. (1996) Effect of capacitation on bull sperm binding to homologous oviductal epithelium. Biol Reprod 54, 575–582.

Lindahl PE & Sjoblom P. (1981) On mechanisms of head-to-head association in bovine spermatozoa. Biol Reprod 25, 29–43.

Luboshitzky R, Shen-Orr Z & Herer P. (2002) Seminal plasma melatonin and gonadal steroids concentrations in normal men. Arch Androl 48, 225–232.

Najafi A, Adutwum E, Yari A, Salehi E, Mikaeili S, Dashtestani F, Abolhassani F, Rashki L, Shiasi S & Asadi E. (2018) Melatonin affects membrane integrity, intracellular reactive oxygen species, caspase3 activity and AKT phosphorylation in frozen thawed human sperm. Cell Tissue Res 372, 149–159.

Nakamura N, Shibata H, O'Brien DA, Mori C & Eddy EM. (2008) Spermatogenic cell-specific type 1 hexokinase is the predominant hexokinase in sperm. Mol Reprod Dev 75, 632–640.

OrtegaFerrusola C,Anel-LopezL, Ortız Rodrıguez JM,Martın MunozP, Alvarez M,de Paz P,Masot J, Redondo E,Balao da SilvaC,Morrell JM, Rodrıguez-Martınez H, Tapia JA, GilMC, Anel L & Pena FJ. (2017) Stallion spermatozoa survivingfreezingand thawingexperiencemembrane depolarization and increased intracellular Na+. Andrology5, 1174–1182.

Perez- Patino C, Barranco I, Casao A, Cebrıan- Perez JA, Muino- Blanco T, Martinez EA & Roca J. (2016) The melatonin concentration in boar seminal plasma: a predictive in vivo fertility marker? Anim Reprod Sci 169, 131.

Petrunkina AM, Waberski D, Bollwein H & Sieme H. (2010) Identifying non-sperm particles during flow cytometric physiological assessment: a simple approach. Theriogenology 73, 995–1000.

Petters RM & Wells KD. (1993) Culture of pig embryos. J Reprod Fertil 48, 61–73.

867 Ramio L, Rivera MM, Ramırez A, Concha II, Pena A, Rigau T & Rodrıguez-Gil JE. (2008) Dynamics
868 of motile-sperm subpopulation structure in boar ejaculates subjected to "in vitro" capacitation and
869 further "in vitro" acrosome reaction. Theriogenology 69, 501–512.

870 Ramio-Lluch L, Fernandez-Novell JM, Pena A, Colas C, Cebrian-Perez JA, Muino-Blanco T,
871 Ramırez A, Concha II, Rigau T & Rodrıguez-Gil JE. (2011) In vitro capacitation and acrosome
872 reaction are concomitant with specific changes in mitochondrial activity in boar sperm: evidence for
873 a nucleated mitochondrial activation and for the existence of a capacitation-sensitive subpopulational
874 structure. Reprod Domest Anim 46, 664–673.

875 Ramio-Lluch L, Yeste M, Fernandez-Novell JM, Estrada E, Rocha L, Cebrian-Perez JA, Muino-
876 Blanco T, Concha II, Ramırez A & Rodrıguez-Gil JE. (2014) Oligomycin A-induced inhibition of
877 mitochondrial ATP-synthase activity suppresses boar sperm motility and in vitro capacitation
878 achievement without modifying overall sperm energy levels. Reprod Fertil Dev 26, 883–897.

879 Rao MV & Gangadharan B. (2008) Antioxidative potential of melatonin against mercury induced
880 intoxication in spermatozoa in vitro. Toxicol In Vitro 22, 935–942.

881 Rath D, Long CR, Dobrinsky JR, Welch GR, Schreier LL & Johnson LA. (1999) In vitro production
882 of sexed embryos for gender preselection: high-speed sorting of X-chromosome-bearing sperm to
883 produce pigs after embryo transfer. J Anim Sci 77, 3346–3352.

884 Rathi R, Colenbrander B, Bevers MM & Gadella BM. (2001) Evaluation of in vitro capacitation of
885 stallion spermatozoa. Biol Reprod 65, 462– 470.

886 Reiter RJ, Tan DX, Osuna C & Gitto E. (2000) Actions of melatonin in the reduction of oxidative
887 stress - A review. J Biomed Sci 7, 444–458.

888 Reiter RJ, Tan DX, Manchester LC, Paredes SD, Mayo JC & Sainz RM. (2009) Melatonin and
889 reproduction revisited. Biol Reprod 81, 445–456.

890 Rodrıguez-Gil JE & Bonet S. (2016) Current knowledge on boar sperm metabolism: comparison with
891 other mammalian species. Theriogenology 85, 4–11.

892 Sarabia L, Maurer I & Bustos-Obregon E. (2009) Melatonin prevents damage elicited by the
893 organophosphorous pesticide diazinon on mouse sperm DNA. Ecotoxicol Environ Saf 72, 663–668.

894 Tait SWG & Green DR. (2012) Mitochondria and cell signalling. J Cell Sci 125, 807–815.

895 Talevi R, Zagami M, Castaldo M & Gualtieri R. (2007) Redox regulation of sperm surface thiols
896 modulates adhesion to the fallopian tube epithelium. Biol Reprod 76, 728–735.

897 Wu JT, Chiang KC & Cheng FP. (2006) Expression of progesterone receptor(s) during capacitation
898 and incidence of acrosome reaction induced by progesterone and zona proteins in boar spermatozoa.
899 Anim Reprod Sci 93, 34–45.

900 Yakirevich EMD & Naota Y. (1999) Characterization of a potent sperm-agglutinating monoclonal
901 antibody and its cognate antigens. Fertil Steril 71, 502–510.

902 Yeste M, Flores E, Estrada E, Bonet S, Rigau T & Rodrıguez-Gil JE. (2013) Reduced glutathione and
903 procaine hydrochloride protect the nucleoprotein structure of boar spermatozoa during freeze-
904 thawing by stabilising disulfide bonds. Reprod Fertil Dev 25, 1036–1050.

905 Yeste M, Estrada E, Pinart E, Bonet S, Miro J & Rodrıguez-Gil JE. (2014) The improving effect of
906 reduced glutathione on boar sperm cryotolerance is related with the intrinsic ejaculate freezability.
907 Cryobiology 68, 251–261.

908 Yeste M, Estrada E, Rocha LG, Marın H, Rodrıguez-Gil JE & Miro J. (2015a) Cryotolerance of
909 stallion sperm is related to ROS production and mitochondrial membrane potential rather than to the
910 integrity of sperm nucleus. Andrology 3, 395–407.

911 Yeste M, Fernandez-Novell JM, Ramio-Lluch L, Estrada E, Rocha LG, Cebrian-Perez JA, Muino-
912 Blanco T, Concha II, Ramırez A & Rodrıguez-Gil JE. (2015b) Intracellular calcium movements of
913 boar spermatozoa during in vitro capacitation and subsequent acrosome exocytosis follow a multiple-
914 storage place, extracellular calcium-dependent model. Andrology 3, 729–747.

915 SUPPORTING INFORMATION

916 Figure S1 Effects of melatonin on the percentage of viability of boar sper-matozoa subjected to in
917 vitro capacitation and subsequent progesterone-induced acrosome exocytosis.

918 Figure S2 Effects of melatonin on the percentage of true acrosome exocy-tosis of boar spermatozoa
919 subjected to in vitro capacitation and subse-quent progesterone-induced acrosome exocytosis.

920 Figure S3 Effects of melatonin on the percentage of cells with capacita-tion-like membrane lipid
921 disorder of boar spermatozoa subjected to in vitro capacitation and subsequent progesterone-induced
922 acrosome exocytosis.

923 Figure S4 Effects of melatonin added at 0 h on tyrosine phosphorylation levels of P32 protein in boar
924 sperm subjected to in vitro capacitation and subsequent progesterone-induced acrosome exocytosis.

925 Figure S5 Effects of the addition of melatonin to CM at 4 h on tyrosine phosphorylation levels of the
926 P32 protein in boar spermatozoa subjected to in vitro capacitation and subsequent progesterone-
927 induced acrosome exocytosis.

928 Figure S6 Images showing the effect of 1 lM melatonin on the formation of cell agglutinations in boar
929 sperm subjected to in vitro capacitation and subsequent progesterone-induced acrosome exocytosis.

930 Figure S7 Effects of melatonin (1 lM) on the distribution of WGA lectin in boar sperm subjected to
931 in vitro capacitation and subsequent proges-terone-induced acrosome exocytosis.

932 Figure S8 Effects of 1 lM melatonin on the distribution of STL lectin in boar sperm subjected to in
933 vitro capacitation and subsequent proges-terone-induced acrosome exocytosis.

934 Figure S9 Effects of 1 lM melatonin on the distribution of PSA lectin in boar sperm subjected to in
935 vitro capacitation and subsequent proges-terone-induced acrosome exocytosis.

936 Figure S10 Effects of 1 lM melatonin on the distribution of PNA lectin in boar sperm subjected to in
937 vitro capacitation and subsequent proges-terone-induced acrosome exocytosis.

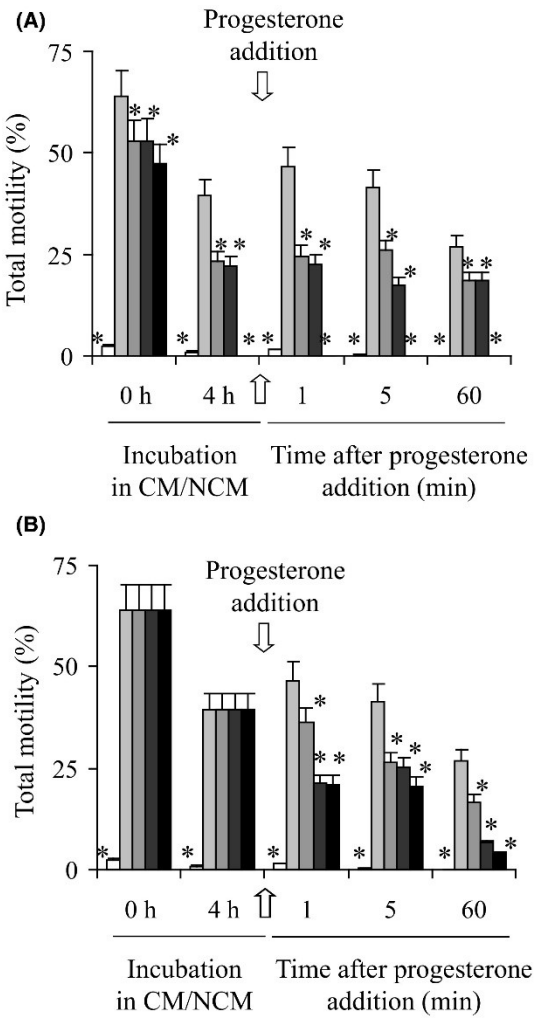
938 Figure S11 Examples of sperm-oocyte complexes considered as monospermic and polyspermic.

939

940

941 Figure 1 Effects of melatonin on total motility of boar spermatozoa sub-jected to in vitro capacitation
942 and subsequent progesterone-induced acro-some exocytosis. (A): Melatonin added at 0 h. (B):
943 Melatonin added together with progesterone at 4 h. White bars: spermatozoa incubated in NCM
944 medium (C). Light grey bars: spermatozoa incubated in CM med-ium (C+). Medium grey bars:
945 spermatozoa incubated in CM added with 0.5 lM melatonin. Dark green bars: spermatozoa incubated
946 in CM added with 1 lM melatonin. Black bars: spermatozoa incubated in CM added with 5 lM
947 melatonin. Asterisks indicate significant ($p < 0.05$) differences between a given treatment and C+
948 samples. Figure shows means SEM for seven separate experiments.

949



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951

952 Table 1. Effects of melatonin on curvilinear velocity (VCL) and average path velocity (VAP) of boar
953 spermatozoa subjected to in vitro capacitation and subsequent, progesterone-induced in vitro
954 acrosome exocytosis

Incubation time	**0 h**	**4 h**	**1 min**	**5 min**	**60 min**
VCL (μm/sec)					
C−	77.0 ± 2.9^{a}*	39.0 ± 1.1^{b}*	39.1 ± 0.9^{b}*	38.5 ± 1.2^{c}*	0^{d}*
C+	65.7 ± 2.1^{a}	72.9 ± 2.9^{b}	74.1 ± 3.2^{b}	81.4 ± 3.7^{b}	81.6 ± 4.3^{b}
0.5 μm melatonin	67.5 ± 2.3^{a}	55.6 ± 1.5^{b}*	57.9 ± 1.7^{b}*	57.0 ± 2.2^{b}*	44.5 ± 1.2^{c}*
1 μm melatonin	56.2 ± 1.8^{a}*	60.7 ± 2.5^{a}*	59.1 ± 2.6^{a}*	61.2 ± 3.5^{a}*	41.2 ± 2.8^{b}*
5 μm melatonin	62.5 ± 2.5^{a}	0^{b}*	0^{b}*	0^{b}*	0^{b}*
0.5 μm melatonin+PG	65.7 ± 2.1^{a}	72.9 ± 2.9^{b}	68.2 ± 2.7^{a}	90.8 ± 4.7^{c}	76.5 ± 3.9^{b}
1 μm melatonin+PG	65.7 ± 2.1^{a}	72.9 ± 2.9^{b}	67.7 ± 2.6^{a}	89.9 ± 4.5^{c}	76.8 ± 4.2^{b}
5 μm melatonin+PG	65.7 ± 2.1^{a}	72.9 ± 2.9^{b}	71.4 ± 3.1^{ab}	81.1 ± 4.3^{c}	66.8 ± 4.0^{a}*
VAP (μm/sec)					
C−	35.5 ± 1.4^{a}*	25.1 ± 1.1^{b}*	27.5 ± 1.6^{b}*	33.6 ± 0.9^{b}*	0^{c}*.
C+	46.0 ± 2.4^{a}	62.6 ± 3.2^{b}	59.8 ± 2.7^{b}	56.7 ± 2.8^{b}	56.8 ± 3.3^{b}
0.5 μm melatonin	48.1 ± 2.0^{a}	44.6 ± 2.2^{a}*	47.0 ± 2.7^{a}*	62.6 ± 3.7^{b}	85.6 ± 5.4^{c}*
1 μm melatonin	39.8 ± 1.4^{a}*	37.4 ± 1.2^{a}*	47.2 ± 2.6^{b}*	67.2 ± 3.6^{c}*	92.4 ± 6.1^{d}*
5 μm melatonin	39.8 ± 1.6^{a}*	0^{b}*	0^{b}*	0^{b}*	0^{b}*
0.5 μm melatonin+PG	46.0 ± 2.4^{a}	62.6 ± 3.2^{b}	40.8 ± 2.0^{a}*	52.2 ± 4.1^{a}	49.1 ± 3.0^{a}
1 μm melatonin+PG	46.0 ± 2.4^{a}	62.6 ± 3.2^{b}	40.9 ± 2.0^{a}*	51.9 ± 3.9^{a}	50.1 ± 3.1^{a}
5 μm melatonin+PG	46.0 ± 2.4^{a}	62.6 ± 3.2^{b}	38.8 ± 1.8^{c}*	35.4 ± 3.1^{c}*	39.5 ± 1.7^{c}*

955 Spermatozoa were subjected to IVC and further IVAE as described in the Materials and Methods
956 section. Determination of motion parameters through CASA and statistical analyses has been also
957 described in the Material and Methods section. Spermatozoa were incubated in a non-capacitating
958 medium (NCM, C−) or in capacitating medium without (CM, C+) or with melatonin at final
959 concentrations of 0.5 μm (0.5 μm melatonin), 1 μm (1 μm melatonin) and 5 μm (5 μm melatonin).
960 After 4 h of incubation, progesterone (PG) was added. Simultaneously, three more aliquots were
961 incubated in capacitating medium and, after 4 h of incubation, were added with progesterone and
962 0.5 μm melatonin (0.5 μm melatonin+PG), progesterone with 1 μm melatonin (1 μm melatonin+PG)
963 and progesterone with 5 μm (5 μm melatonin +PG). In all cases, spermatozoa were subsequently
964 incubated, and aliquots were taken after 1, 5 and 60 min of progesterone addition. Different
965 superscript letters (a–d) indicate significant differences ($p < 0.05$) between columns within a given
966 row. Asterisks indicate significant differences ($p < 0.05$) when compared with C+ (CM) at the same
967 time point. Results are shown as means ± SEM for seven separate experiments.

968

969 Table 2. Effects of melatonin on linearity (LIN) and straightness (STR) coefficients of boar
970 spermatozoa subjected to in vitro capacitation and subsequent, progesterone-induced in vitro
971 acrosome exocytosis

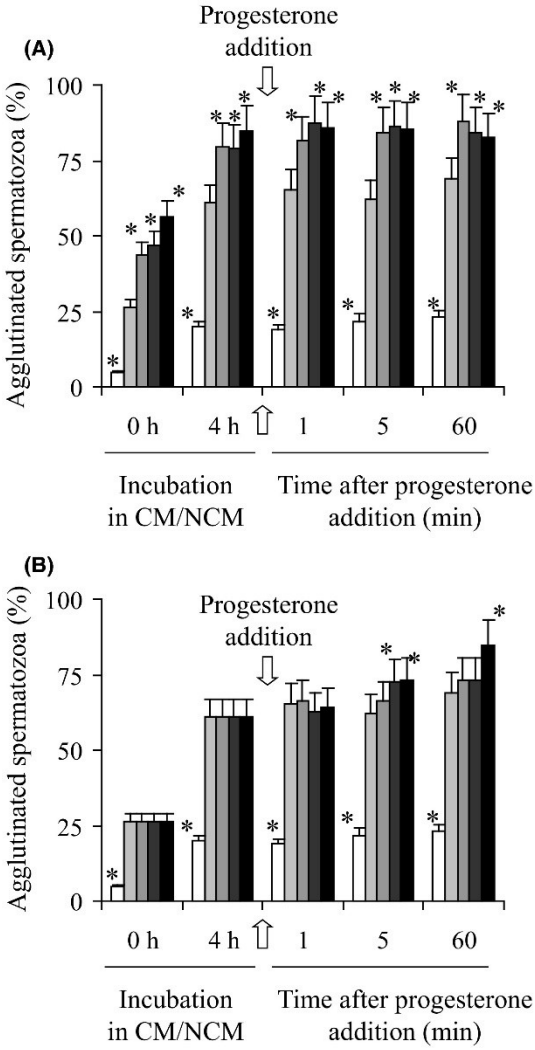
Incubation time	**0 h**	**4 h**	**1 min**	**5 min**	**60 min**
LIN (%)					
C−	23.6 ± 1.1^{a}*	35.1 ± 1.3^{b}*	36.3 ± 1.6^{b}	33.6 ± 1.5^{b}*	0^{c}*
C+	36.2 ± 1.4^{a}	44.8 ± 2.4^{b}	39.9 ± 1.7^{a}	47.9 ± 2.3^{b}	37.1 ± 2.0^{a}
0.5 µm melatonin	42.0 ± 1.9^{a}	34.8 ± 1.8^{b}*	33.5 ± 1.6^{b}	43.2 ± 2.2^{a}	33.2 ± 2.1^{b}
1 µm melatonin	36.2 ± 1.7^{a}	30.8 ± 2.1^{a}*	33.6 ± 1.4^{a}	44.9 ± 2.4^{b}	35.3 ± 1.8^{a}
5 µm melatonin	39.8 ± 2.2^{a}	0^{b}*	0^{b}*	0^{b}*	0^{b}*
0.5 µm melatonin+PG	36.2 ± 1.4^{a}	44.8 ± 2.4^{b}	26.8 ± 1.2^{c}*	36.5 ± 1.8^{a}*	32.7 ± 1.5^{a}
1 µm melatonin+PG	36.2 ± 1.4^{a}	44.8 ± 2.4^{b}	25.7 ± 1.6^{c}*	36.3 ± 1.9^{a}*	33.2 ± 1.8^{a}
5 µm melatonin+PG	36.2 ± 1.4^{a}	44.8 ± 2.4^{b}	26.0 ± 1.3^{c}*	30.1 ± 1.9^{c}*	28.2 ± 1.2^{c}*
STR (%)					
C−	55.8 ± 2.3^{a}*	48.5 ± 2.4^{b}*	44.6 ± 2.0^{b}*	43.6 ± 1.9^{b}*	0^{c}*
C+	63.3 ± 2.4^{a}	70.9 ± 3.0^{b}	67.7 ± 2.8^{ab}	71.0 ± 2.9^{b}	76.2 ± 3.3^{b}
0.5 µm melatonin	64.7 ± 2.3^{a}	83.9 ± 3.4^{b}*	67.1 ± 3.0^{a}	69.0 ± 3.6^{a}	81.2 ± 4.5^{b}
1 µm melatonin	62.1 ± 2.4^{a}	52.1 ± 1.5^{b}*	68.6 ± 2.5^{ab}	79.1 ± 3.6^{c}	73.9 ± 3.1^{b}
5 µm melatonin	63.1 ± 2.4^{a}	0^{b}*	0^{b}*	0^{b}*	0^{b}*
0.5 µm melatonin+PG	63.3 ± 2.4^{a}	70.9 ± 3.0^{b}	60.6 ± 2.5^{a}	76.1 ± 3.7^{b}	77.1 ± 4.4^{b}
1 µm melatonin+PG	63.3 ± 2.4^{a}	70.9 ± 3.0^{b}	58.1 ± 1.8^{a}	70.5 ± 2.6^{b}	76.0 ± 4.5^{b}
5 µm melatonin+PG	63.3 ± 2.4^{a}	70.9 ± 3.0^{b}	55.6 ± 1.6^{b}*	64.6 ± 1.9^{a}*	64.8 ± 2.9^{a}*

972 Spermatozoa were subjected to IVC and further IVAE as described in the Material and Methods
973 section. Determination of motion parameters through CASA and statistical analyses has been also
974 described in the Material and Methods section. Spermatozoa were incubated in a non-capacitating
975 medium (NCM, C−) or in capacitating medium without (CM, C+) or with melatonin at final
976 concentrations of 0.5 µm (0.5 µm Melatonin), 1 µm (1 µm melatonin) and 5 µm (5 µm Melatonin).
977 After 4 h of incubation, progesterone (PG) was added. Simultaneously, three more aliquots were
978 incubated in capacitating medium and, after 4 h of incubation, were added with progesterone and
979 0.5 µm melatonin (0.5 µm melatonin+PG), progesterone with 1 µm melatonin (1 µm melatonin+PG)
980 and progesterone with 5 µm (5 µm melatonin +PG). In all cases, spermatozoa were subsequently
981 incubated, and aliquots were taken after 1, 5 and 60 min of progesterone addition. Different
982 superscript letters (a–c) indicate significant differences ($p < 0.05$) between columns within a given
983 row. Asterisks indicate significant differences ($p < 0.05$) when compared with C+ (CM) at the same
984 time point. Results are shown as means ± SEM for seven separate experiments.

985

Figure 2

Effects of melatonin on the percentage of agglutinated cells of boar spermatozoa subjected to in vitro capacitation and subsequent progesterone-induced acrosome exocytosis. (A): Melatonin added at 0 h. (B): Melatonin added together with progesterone at 4 h. White bars: spermatozoa incubated in NCM medium (C−). Light grey bars: spermatozoa incubated in CM medium (C+). Medium grey bars: spermatozoa incubated in CM added with 0.5 µm melatonin. Dark green bars: spermatozoa incubated in CM added with 1 µm melatonin. Black bars: spermatozoa incubated in CM added with 5 µm melatonin. Asterisks indicate significant ($p < 0.05$) differences between a given treatment and C+ samples. Figure shows means ± SEM for seven separate experiments.



997 **Figure 3**

998 Effects of melatonin on the percentage of agglutinated cells with beating tails of boar spermatozoa
999 subjected to in vitro capacitation and subsequent progesterone-induced acrosome exocytosis. (A):
1000 Melatonin added at 0 h. (B): Melatonin added together with progesterone at 4 h. White bars:
1001 spermatozoa incubated in NCM medium (C−). Light grey bars: spermatozoa incubated in CM
1002 medium (C+). Medium grey bars: spermatozoa incubated in CM added with 0.5 μm melatonin. Dark
1003 green bars: spermatozoa incubated in CM added with 1 μm melatonin. Black bars: spermatozoa
1004 incubated in CM added with 5 μm melatonin. Asterisks indicate significant ($p < 0.05$) differences
1005 between a given treatment and C+ samples. Figure shows means ± SEM for seven separate
1006 experiments.

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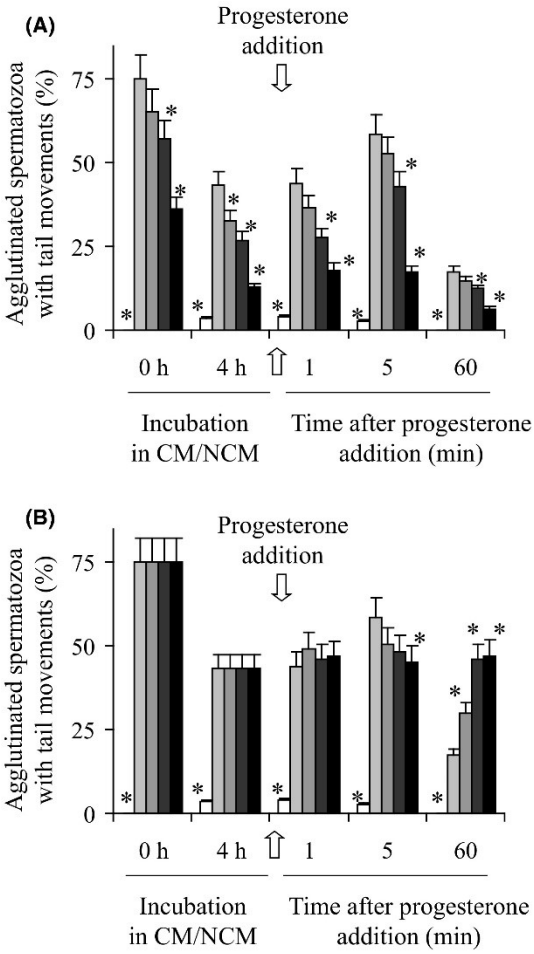

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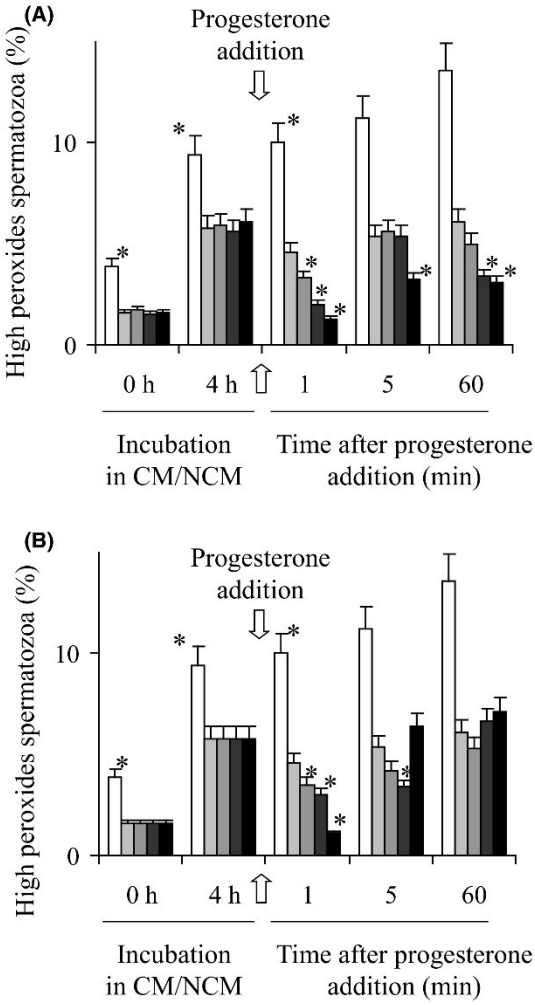
1010 **Figure 4**

1011 Effects of melatonin on the percentage of viable spermatozoa with high intracellular peroxide levels
1012 of boar spermatozoa subjected to in vitro capacitation and subsequent progesterone-induced
1013 acrosome exocytosis. (A): Melatonin added at 0 h. (B): Melatonin added together with progesterone
1014 at 4 h. White bars: spermatozoa incubated in NCM medium (C−). Light grey bars: spermatozoa
1015 incubated in CM medium (C+). Medium grey bars: spermatozoa incubated in CM added with 0.5 µm
1016 melatonin. Dark green bars: spermatozoa incubated in CM added with 1 µm melatonin. Black bars:
1017 spermatozoa incubated in CM added with 5 µm melatonin. Asterisks indicate significant ($p < 0.05$)
1018 differences between a given treatment and C+ samples. Figure shows means ± SEM for seven
1019 separate experiments.



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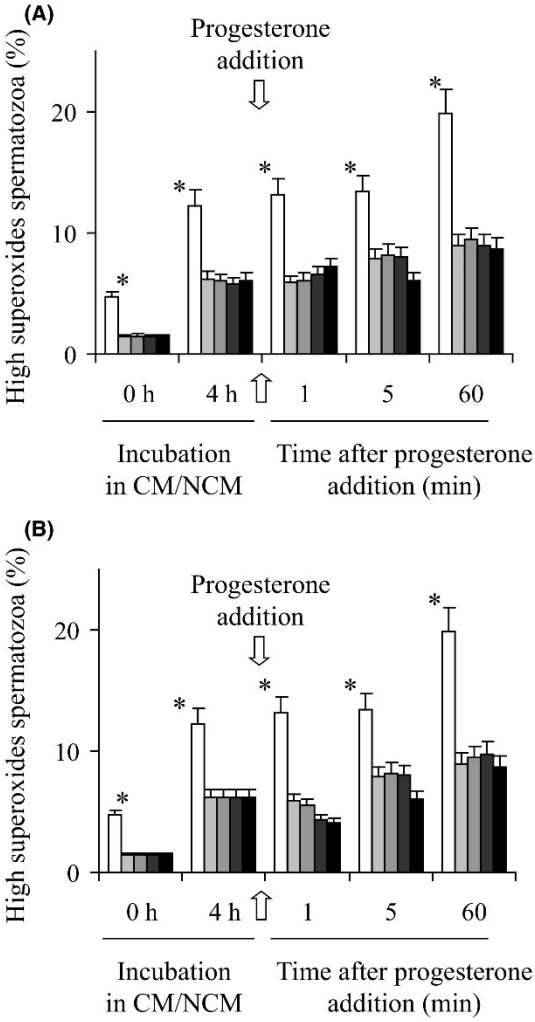
1021

1022 **Figure 5**

1023 Effects of melatonin on the percentage of viable spermatozoa with high intracellular superoxide levels
1024 of boar spermatozoa subjected to in vitro capacitation and subsequent progesterone-induced
1025 acrosome exocytosis. (A): Melatonin added at 0 h. (B): Melatonin added together with progesterone
1026 at 4 h. White bars: spermatozoa incubated in NCM medium (C−). Light grey bars: spermatozoa
1027 incubated in CM medium (C+). Medium grey bars: spermatozoa incubated in CM added with 0.5 µm
1028 melatonin. Dark green bars: spermatozoa incubated in CM added with 1 µm melatonin. Black bars:
1029 spermatozoa incubated in CM added with 5 µm melatonin. Asterisks indicate significant ($p < 0.05$)
1030 differences between a given treatment and C+ samples. Figure shows means ± SEM for seven
1031 separate experiments.

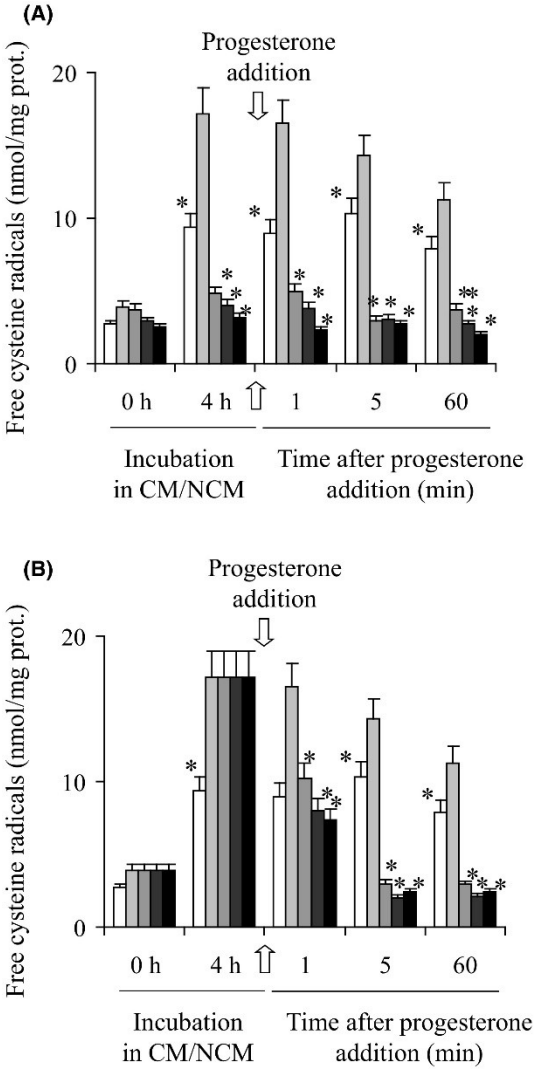


1032

1033

1034 **Figure 6**

1035 Effects of melatonin on the head intracellular free cysteine radicals levels of boar spermatozoa
1036 subjected to in vitro capacitation and subsequent progesterone-induced acrosome exocytosis. (A):
1037 Melatonin added at 0 h. (B): Melatonin added together with progesterone at 4 h. White bars:
1038 spermatozoa incubated in NCM medium (C−). Light grey bars: spermatozoa incubated in CM
1039 medium (C+). Medium grey bars: spermatozoa incubated in CM added with 0.5 μm melatonin. Dark
1040 green bars: spermatozoa incubated in CM added with 1 μm melatonin. Black bars: spermatozoa
1041 incubated in CM added with 5 μm melatonin. Asterisks indicate significant ($p < 0.05$) differences
1042 between a given treatment and C+ samples. Figure shows means ± SEM for seven separate
1043 experiments.

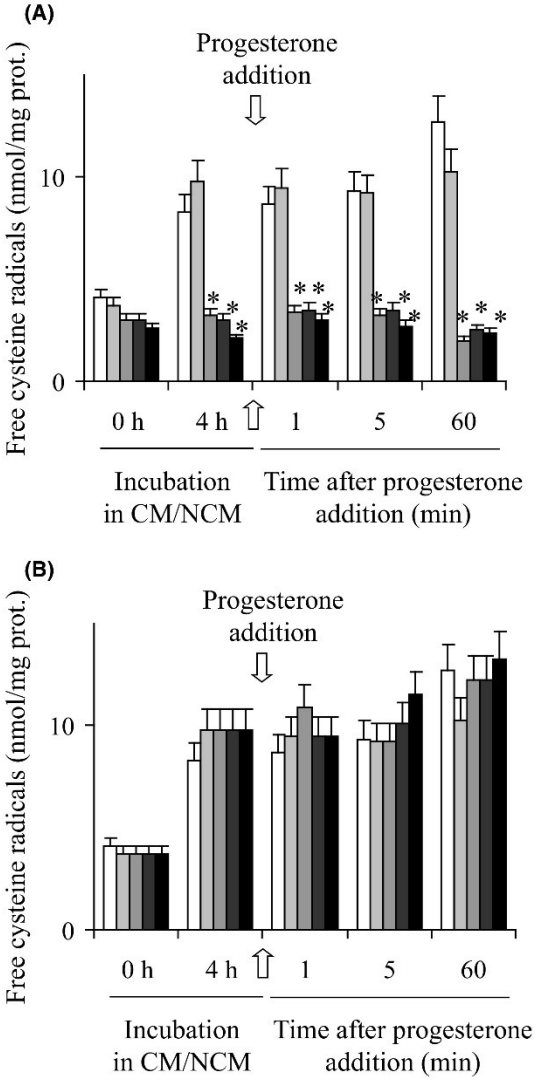


1044

1045

1046 **Figure 7**

1047 Effects of melatonin on the tail intracellular free cysteine radical levels of boar spermatozoa subjected
1048 to in vitro capacitation and subsequent progesterone-induced acrosome exocytosis. (A): Melatonin
1049 added at 0 h. (B): Melatonin added together with progesterone at 4 h. White bars: spermatozoa
1050 incubated in NCM medium (C−). Light grey bars: spermatozoa incubated in CM medium (C+).
1051 Medium grey bars: spermatozoa incubated in CM added with 0.5 µm melatonin. Dark green bars:
1052 spermatozoa incubated in CM added with 1 µm melatonin. Black bars: spermatozoa incubated in CM
1053 added with 5 µm melatonin. Asterisks indicate significant ($p < 0.05$) differences between a given
1054 treatment and C+ samples. Figure shows means ± SEM for seven separate experiments.



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1056

1057 Table 3. Effects of melatonin on mean amplitude of lateral head displacement (ALH) and frequency
1058 of head displacement (BCF) of boar spermatozoa subjected to in vitro capacitation and subsequent,
1059 progesterone-induced in vitro acrosome exocytosis

Incubation time	**0 h**	**4 h**	**1 min**	**5 min**	**60 min**
ALH (μm)					
C−	$2.56 \pm 0.05^{a*}$	$2.57 \pm 0.09^{a*}$	$2.18 \pm 0.11^{b*}$	$2.14 \pm 0.10^{b*}$	0^{c*}
C+	3.80 ± 0.12^{a}	4.13 ± 0.13^{b}	4.93 ± 0.15^{c}	4.64 ± 0.15^{c}	4.00 ± 0.11^{b}
0.5 μm melatonin	$3.31 \pm 0.09^{a*}$	$2.88 \pm 0.08^{b*}$	$4.10 \pm 0.12^{c*}$	$3.63 \pm 0.12^{ac*}$	$5.13 \pm 0.21^{d*}$
1 μm melatonin	3.50 ± 0.14^{a}	$2.72 \pm 0.09^{b*}$	$3.68 \pm 0.08^{a*}$	$3.49 \pm 0.11^{a*}$	3.85 ± 0.10^{a}
5 μm Melatonin	3.89 ± 0.13^{a}	0^{b*}	0^{b*}	0^{b*}	0^{b*}
0.5 μm melatonin+PG	3.80 ± 0.12^{a}	4.13 ± 0.13^{b}	4.82 ± 0.20^{b}	5.09 ± 0.20^{b}	3.81 ± 0.14^{a}
1 μm melatonin+PG	3.80 ± 0.12^{a}	4.13 ± 0.13^{b}	5.38 ± 0.25^{c}	4.96 ± 0.19^{c}	$3.09 \pm 0.11^{d*}$
5 μm melatonin+PG	3.80 ± 0.12^{a}	4.13 ± 0.13^{b}	5.18 ± 0.20^{c}	4.93 ± 0.18^{c}	$2.99 \pm 0.06^{d*}$
BCF (Hz)					
C−	$2.92 \pm 0.04^{a*}$	$3.97 \pm 0.26^{b*}$	$3.88 \pm 0.21^{b*}$	$4.01 \pm 0.24^{b*}$	0^{c*}
C+	6.68 ± 0.17^{a}	6.54 ± 0.19^{a}	7.18 ± 0.18^{b}	6.28 ± 0.21^{a}	6.03 ± 0.19^{c}
0.5 μm melatonin	6.73 ± 0.15^{a}	6.70 ± 0.16^{a}	7.20 ± 0.12^{b}	$6.97 \pm 0.24^{b*}$	$6.85 \pm 0.21^{ab*}$
1 μm melatonin	$6.16 \pm 0.11^{a*}$	$5.95 \pm 0.11^{a*}$	$6.76 \pm 0.20^{b*}$	$6.90 \pm 0.24^{b*}$	$7.20 \pm 0.27^{c*}$
5 μm melatonin	$6.05 \pm 0.10^{a*}$	0^{b*}	0^{b*}	0^{b*}	0^{b*}
0.5 μm melatonin+PG	6.68 ± 0.17^{a}	6.54 ± 0.19^{a}	6.86 ± 0.21^{a}	$5.46 \pm 0.09^{b*}$	$4.37 \pm 0.06^{c*}$
1 μm melatonin+PG	6.68 ± 0.17^{a}	6.54 ± 0.19^{a}	7.48 ± 0.29^{b}	$4.98 \pm 0.09^{c*}$	$3.91 \pm 0.08^{d*}$
5 μm melatonin+PG	6.68 ± 0.17^{a}	6.54 ± 0.19^{ab}	$6.44 \pm 0.17^{b*}$	$4.92 \pm 0.08^{c*}$	$3.85 \pm 0.07^{d*}$

1060 Spermatozoa were subjected to IVC and further IVAE as described in the Material and Methods
1061 section. Determination of motion parameters through CASA and statistical analyses has been also
1062 described in the Material and Methods section. Spermatozoa were incubated in a non-capacitating
1063 medium (NCM, C−) or in capacitating medium without (CM, C+) or with melatonin at final
1064 concentrations of 0.5 μm (0.5 μm melatonin), 1 μm (1 μm melatonin) and 5 μm (5 μm melatonin).
1065 After 4 h of incubation, progesterone (PG) was added. Simultaneously, three more aliquots were
1066 incubated in capacitating medium and, after 4 h of incubation, were added with progesterone and
1067 0.5 μm melatonin (0.5 μm melatonin+PG), progesterone with 1 μm melatonin (1 μm melatonin+PG)
1068 and progesterone with 5 μm (5 μm melatonin +PG). In all cases, spermatozoa were subsequently
1069 incubated, and aliquots were taken after 1, 5 and 60 min of progesterone addition. Different
1070 superscript letters (a–c) indicate significant differences ($p < 0.05$) between columns within a given
1071 row. Asterisks indicate significant differences ($p < 0.05$) when compared with C+ (CM) at the same
1072 time point. Results are shown as means ± SEM for seven separate experiments.

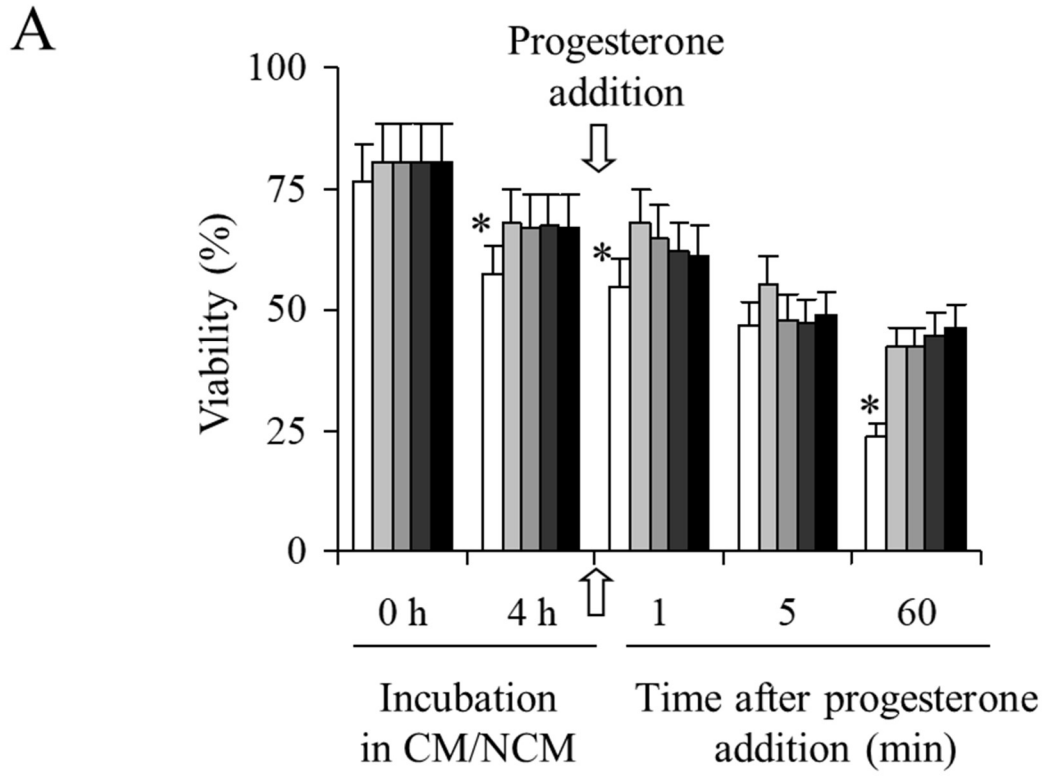
1073

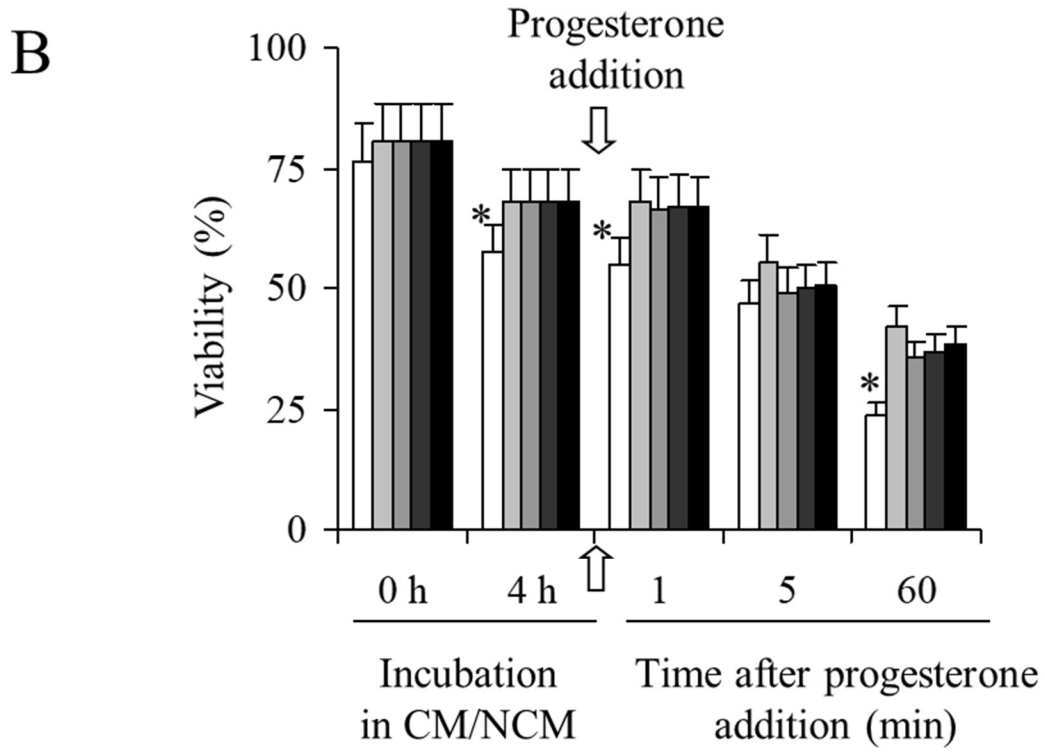
1074 Table 4. Effects of melatonin on the adherence and penetration abilities of boar spermatozoa subjected
1075 to co-incubation with in vitro maturated porcine oocytes

	Control (*n* = 53)	**Capacitation with 1 µm melatonin (*n* = 55)**	**Co-incubation with 1 µm melatonin (*n* = 57)**
Adhered spermatozoa/oocyte	78.4 ± 1.8[a]	68.2 ± 2.7[b]	88.9 ± 1.7[c]
Total penetration rate (%)	90.6[a]	89.1[a]	93.0[a]
Monospermy (%)	69.8[a]	74.5[a]	70.2[a]
Polyspermy (%)	20.8[a]	14.5[b]	22.8[a]

1076 Control: Spermatozoa subjected to a previous standard in vitro capacitation procedure through
1077 incubation at 38.5 °C and 5% CO_2 for 4 h. Capacitation with 1 µm melatonin: Spermatozoa subjected
1078 to in vitro capacitation in a medium added with 1 µm melatonin. Co-incubation with 1 µm melatonin:
1079 Spermatozoa subjected to a previous standard in vitro capacitation procedure for 4 h. Melatonin at
1080 1 µm was added when spermatozoa and oocytes were co-incubated. Different superscript letters
1081 between columns within a given row indicate significant ($p < 0.05$) differences between groups.

1082





Suppl. Fig. 1

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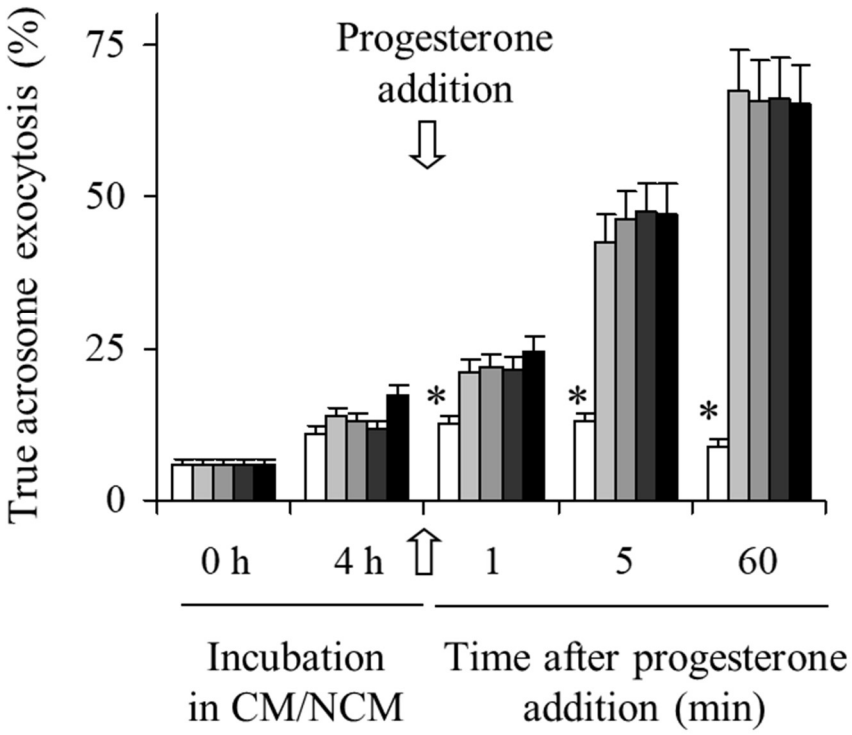
A



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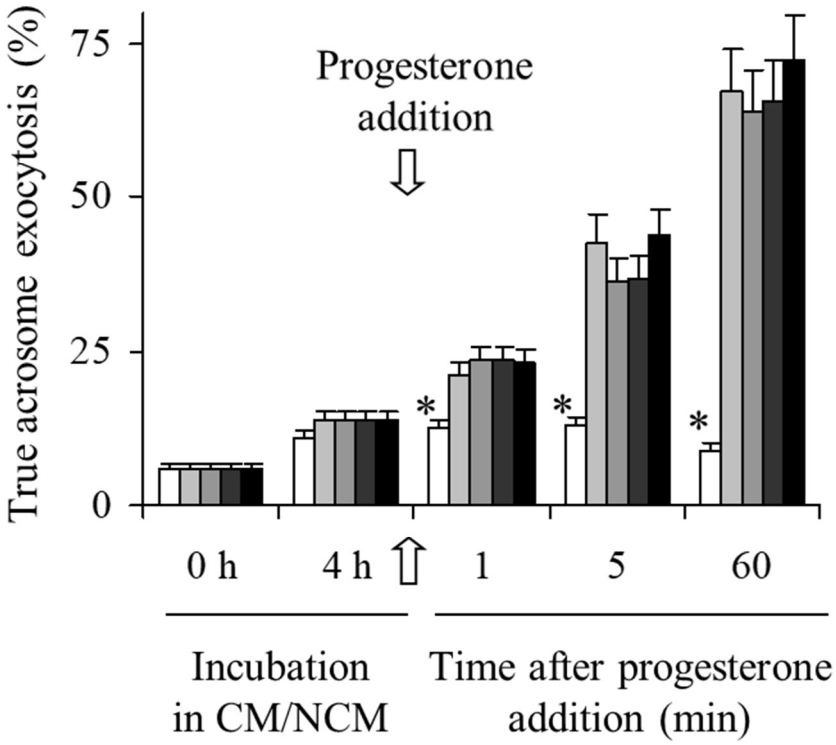

Suppl. Fig. 2

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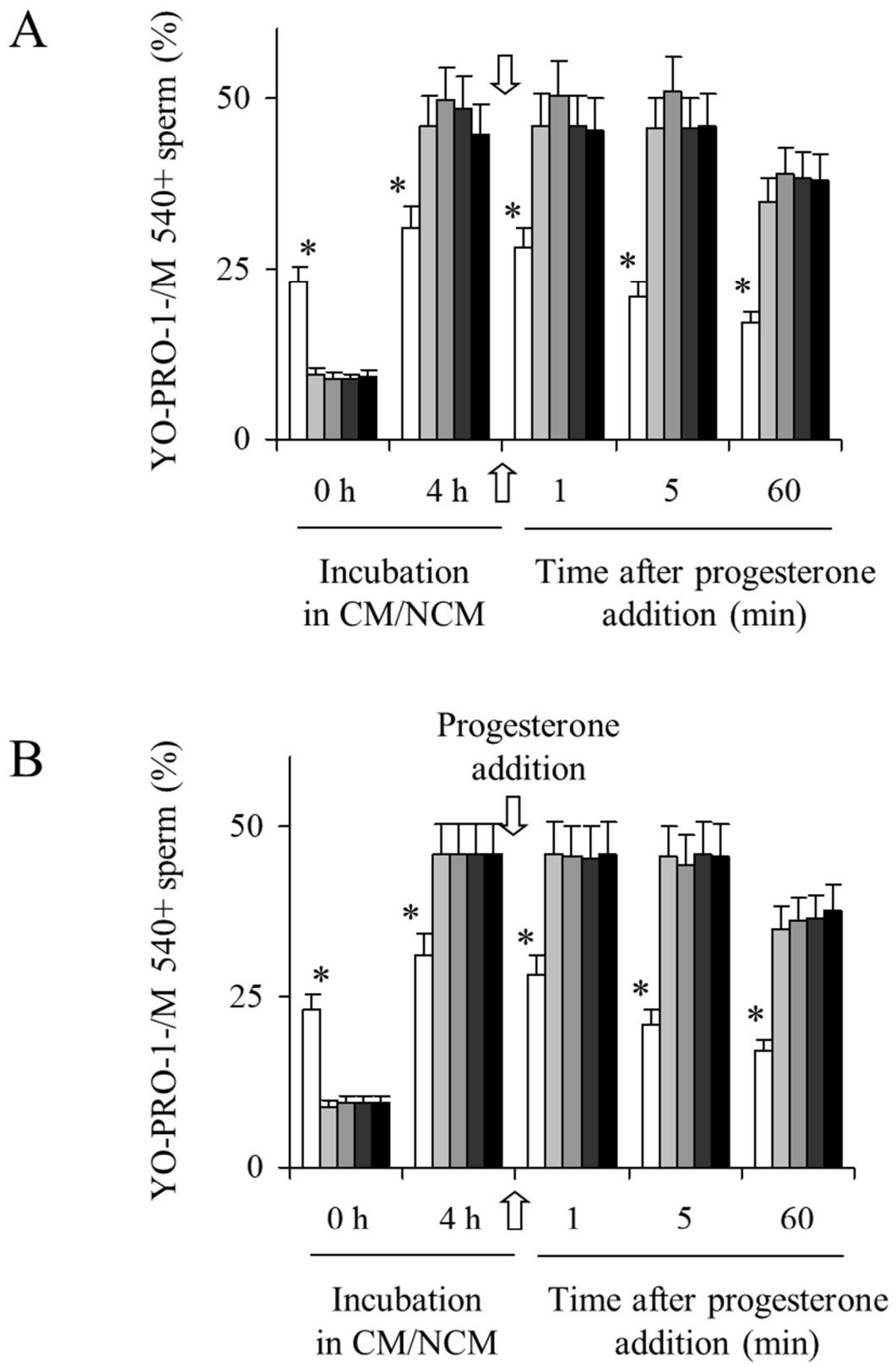

Suppl. Fig. 3

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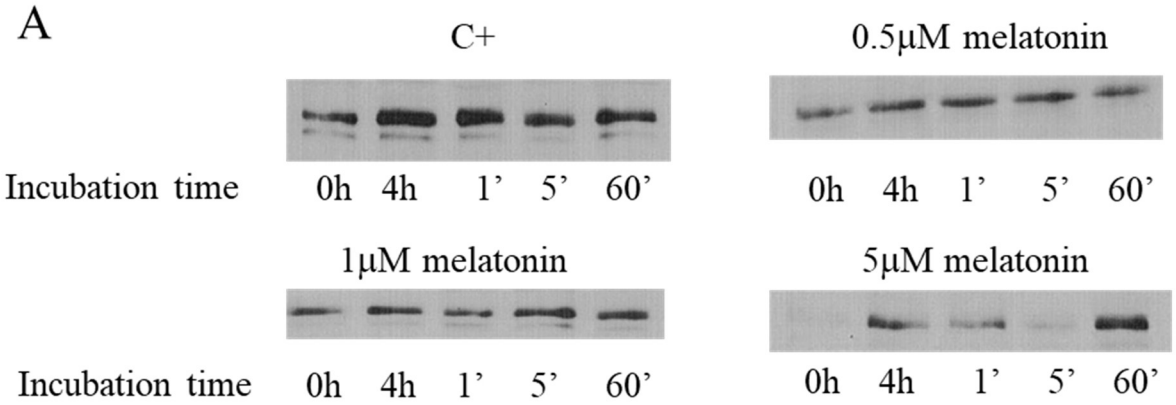

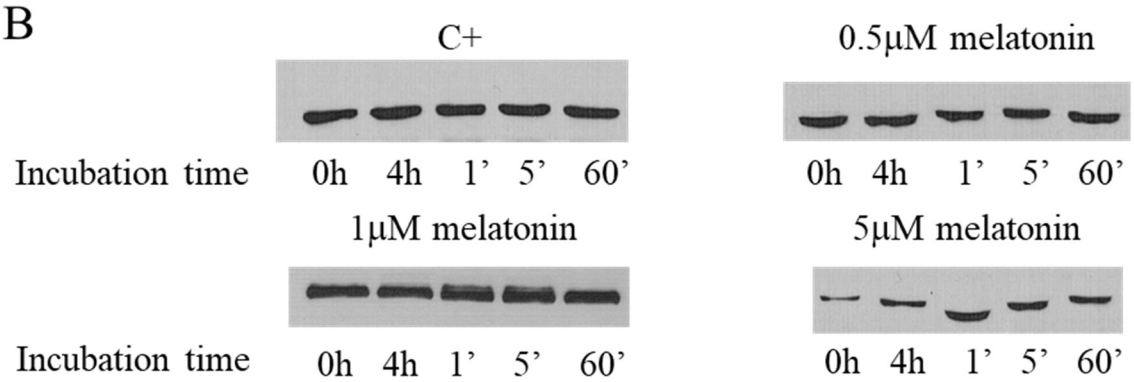

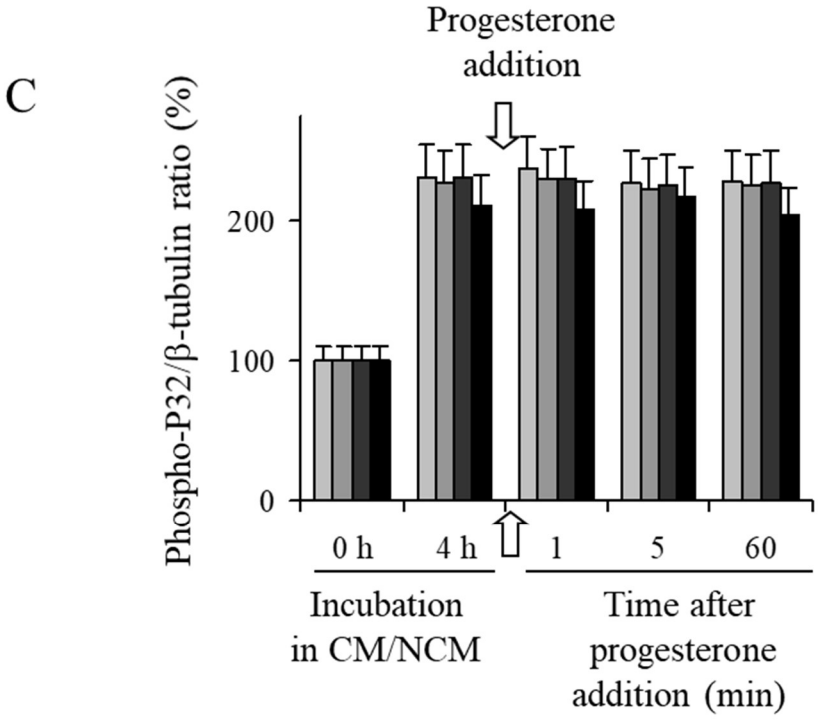

Suppl. Fig. 4

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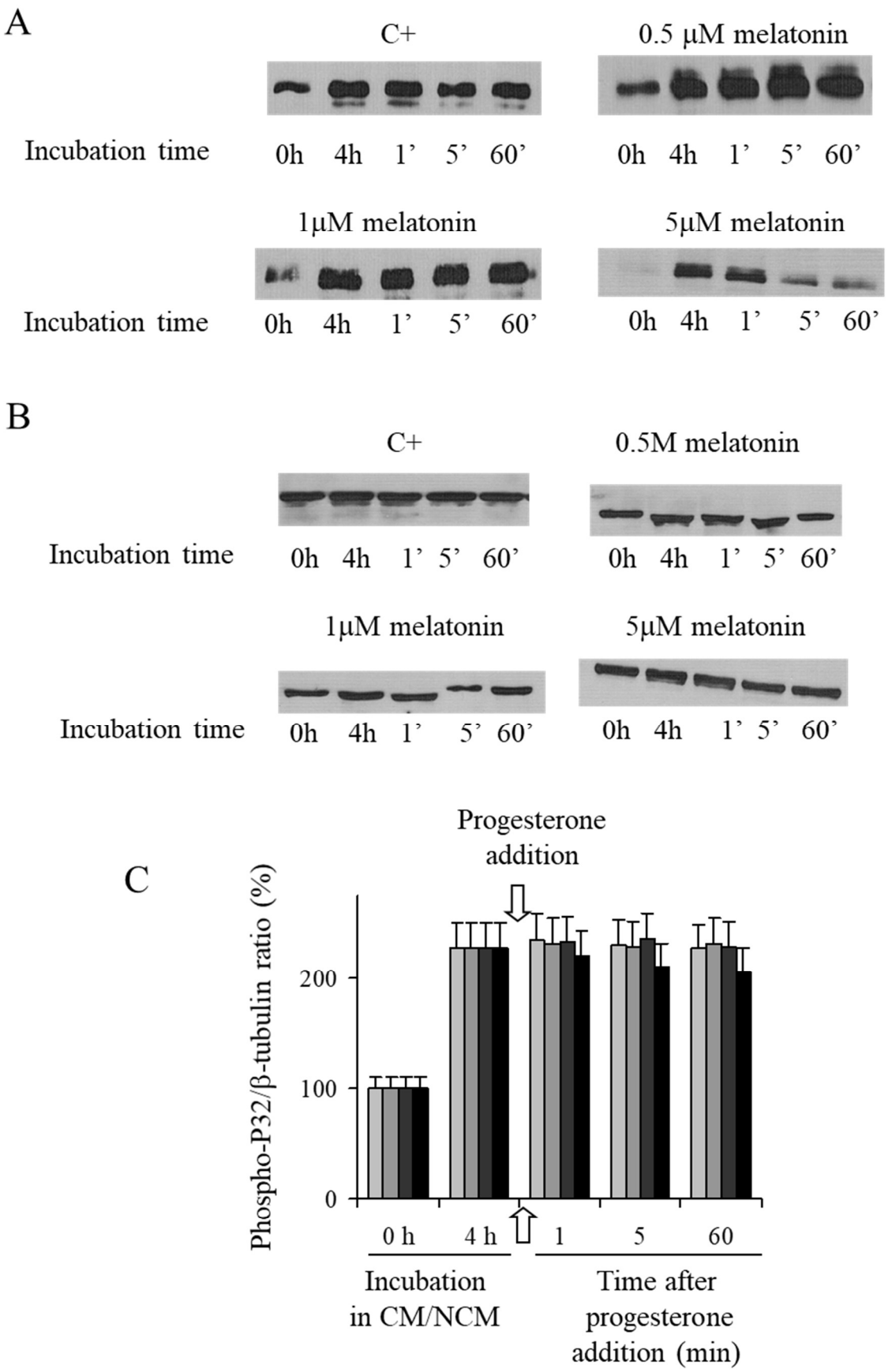

Suppl. Fig. 5

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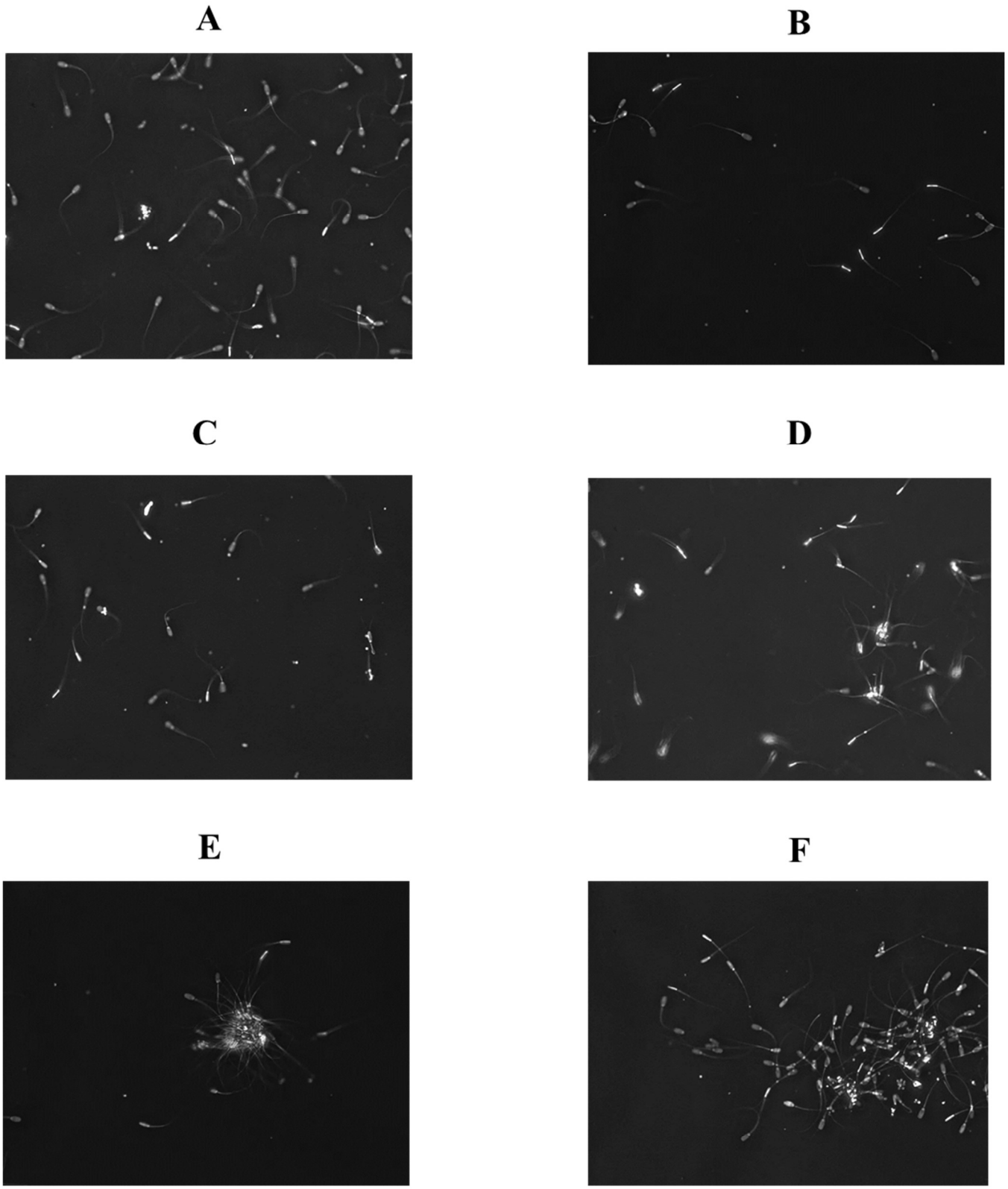

Suppl. Fig. 6

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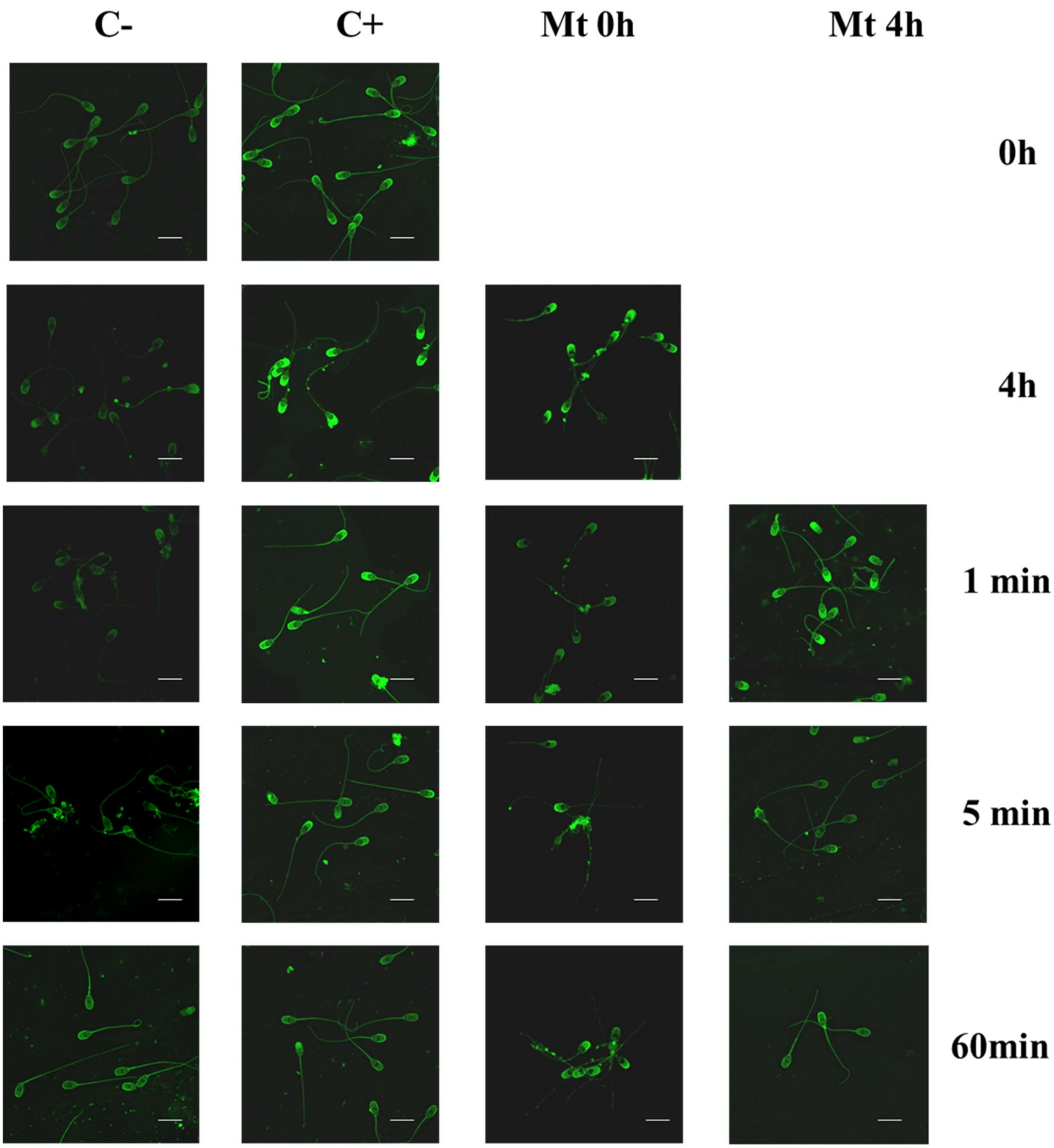

Suppl. Fig. 7

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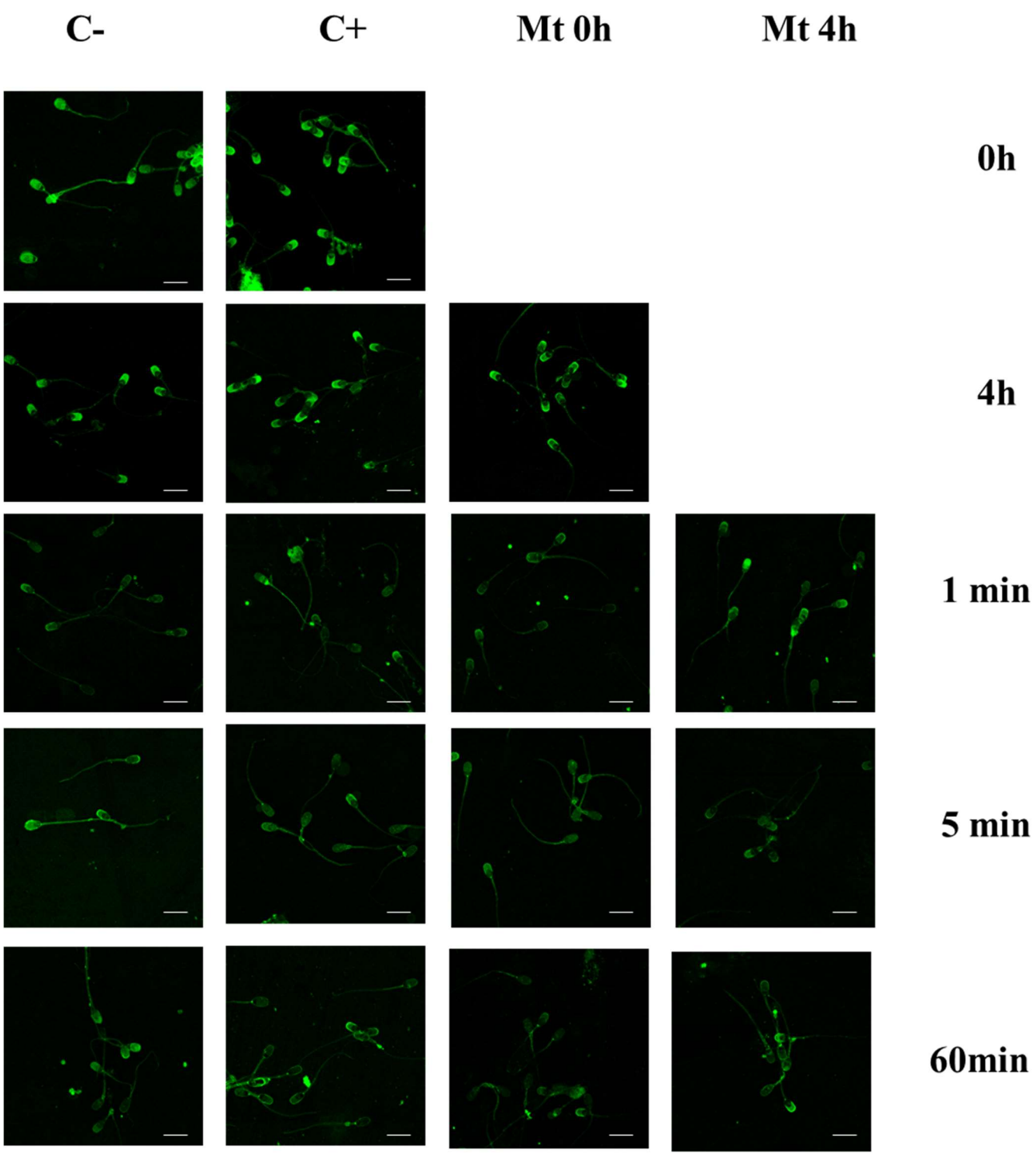

Suppl. Fig. 8

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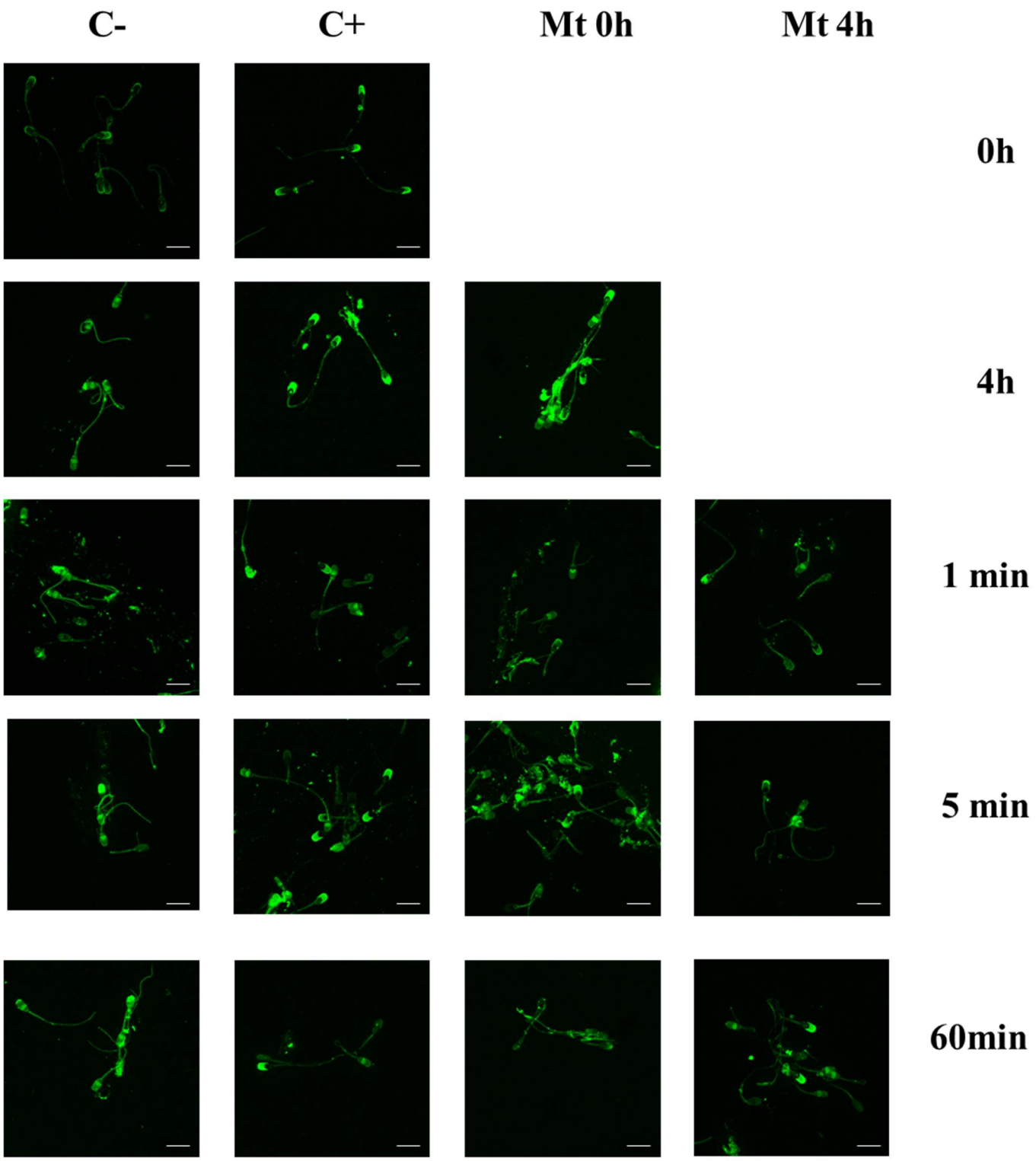

Suppl. Fig. 9

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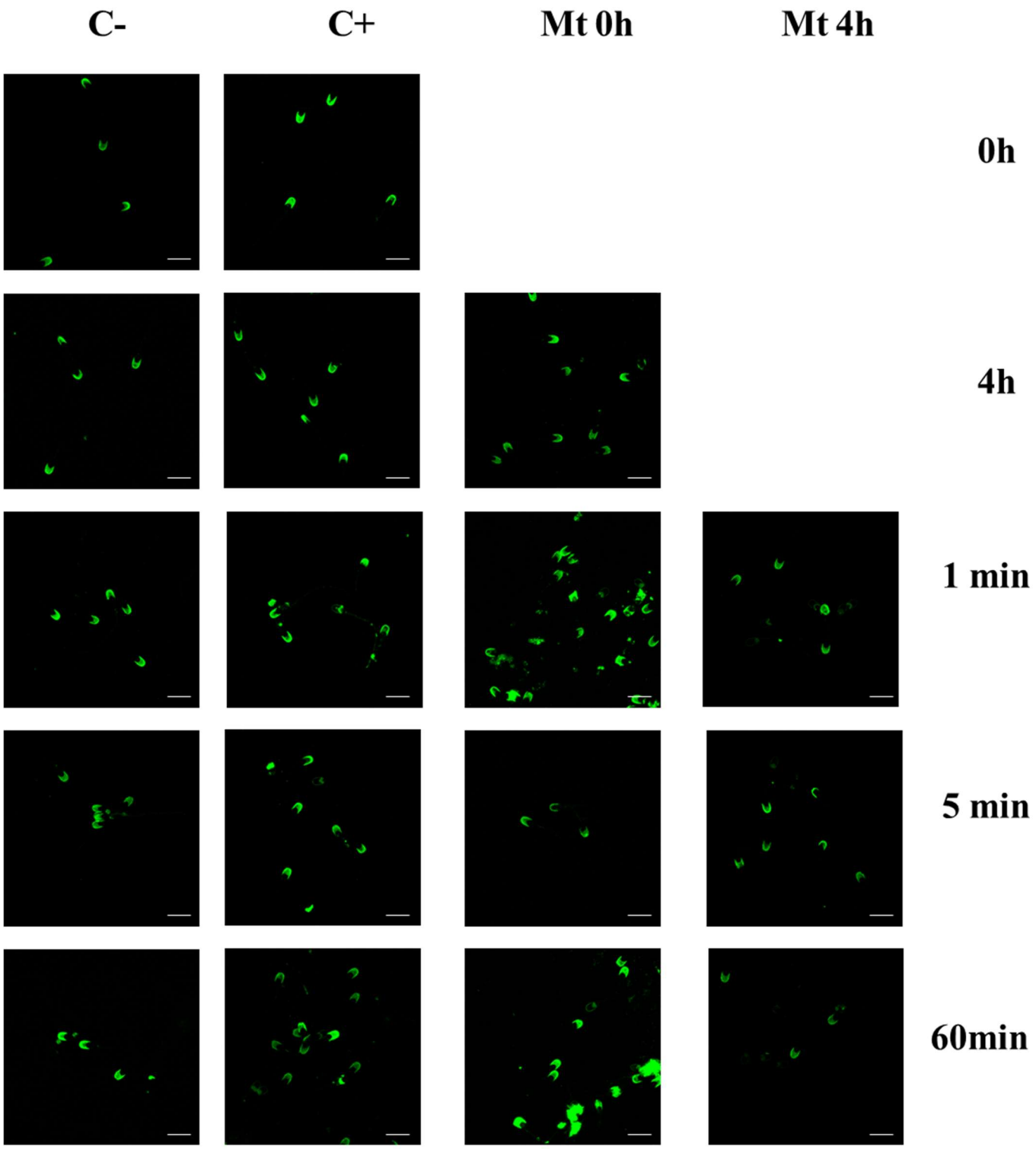

Suppl. Fig. 10

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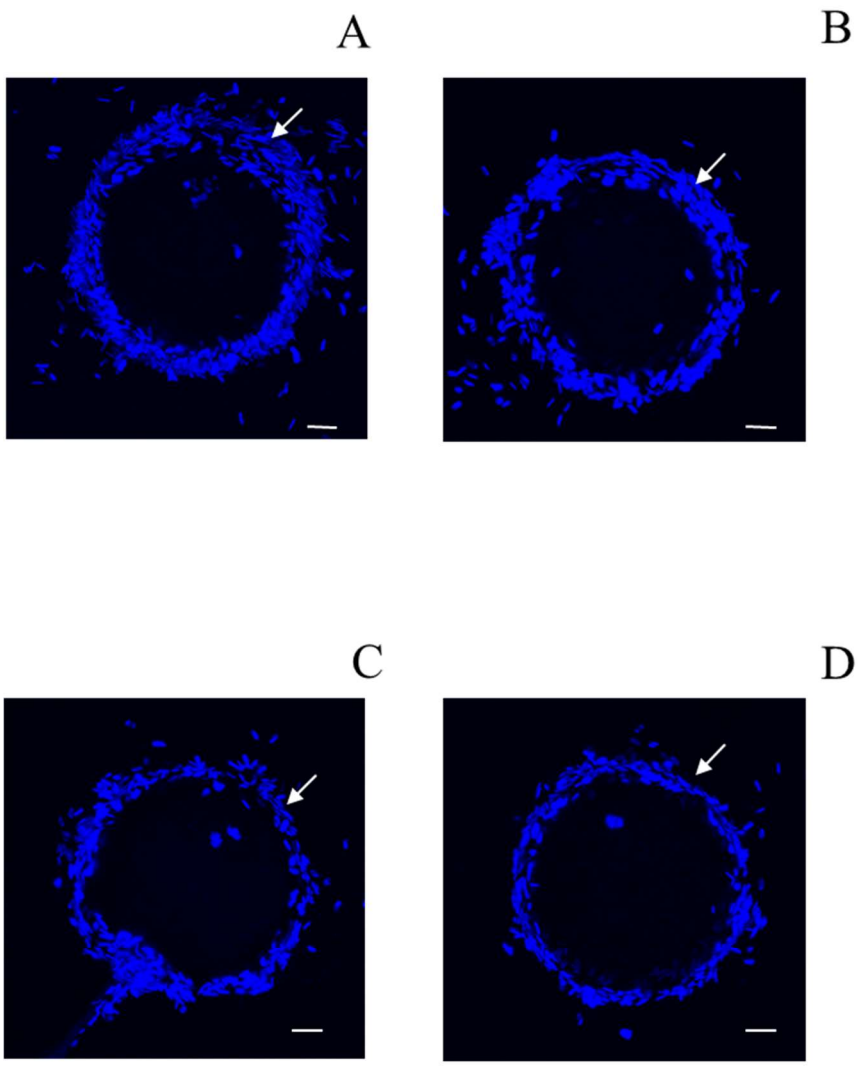

Suppl. Fig. 11

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